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Attempts to Increase Resistance of Mice to Bacterial Infection by Prolonged Low Dose γ Irradiation.* (25989)

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Previous experiments have shown that prolonged daily exposure to γ radiation increased susceptibility of mice to bacterial infection, as determined by challenge with *Pseudomonas aeruginosa,* and that the increase was proportional to dose rate rather than total amount of radiation accumulated(1). Even at the rate of 15 r/d a slight increase in susceptibility was detectable after 9-15 weeks exposure (2). The literature, however, contains reports(3,4) which suggest the possibility that at very low dose rates an opposite effect may result, *i.e.* that prolonged daily exposure to very small doses may actually increase resistance to bacterial infection. For if one assumes, as most investigators do, that the occasional death which occurs among stock laboratory animals is probably caused by intercurrent infection, one may reasonably conclude that any reduction in mortality among irradiated animals below that of their controls may well be due to a lower incidence of death from intercurrent infection, hence to increased resistance to latent infection. Pertinent to this line of reasoning are the following observations: Lorenz *et al.* in an early attempt to establish limits of "permissible" daily doses of ionizing radiation exposed mature laboratory animals to 5 levels (8.8-.11 r) of γ radiation daily until death. Although the life span of those receiving the 3 highest doses was shortened, mortality of those receiving 1.1 r/day was slightly lower and of those receiving .11 r considerably lower than that of their controls during the first 23 months of exposure(3). Carlson, Scheyer, and Jackson found the mean survival time of rats exposed to 0.8 r/day to be longer than that of their unirradiated controls whether their environmental temperatures were maintained at 25° or 5°C(4). The present experiments were undertaken in an attempt to detect any increased resistance to an experimental bacterial

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TABLE I. Comparison of Mortalities of Irradiated and Control Mice in Each Challenge Inoculation.

	Dose rate, r/day		
	.5	1	2
	Wk of exposure		
No difference between irradiated and control mice	27	4	14
	30	5	22
	32	9	27
		17	30
		26	
		27	
		30	
Slightly lower among irradiated		26	23
		27	
		30	
		39	
Slightly higher among irradiated		18	
		24	
		27	
		31	

infection among mice exposed to 0.5, 1.0 or 2.0 r per day for various lengths of time. Preliminary experiments did in fact give evidence of slightly increased resistance but additional experiments failed to confirm earlier results.

Materials and methods. Mice. CF-1 females 3 weeks old on arrival in the laboratory were kept under observation for one week, then randomly distributed into groups of 10, half of them to be irradiated and half kept as controls. They were housed in Lucite cages measuring 7½ x 12 x 7 inches covered with perforated aluminum tops. Rockland mouse pellets were available at all times. Tap water was supplied in sterilized bottles which were changed every day. Cages were changed and disinfected once a week. *Irradiation* was carried out in an air conditioned room in the subbasement of the Argonne Cancer Research Hospital.† Mice were exposed 6 days a week to γ radiation from a nominal 0.5 curie Co^{60} source at 3 dose rates: 0.5, 1.0 and 2.0 r/day. Cages were placed on curved wooden racks the center of each at its appropriate distance from the source. The daily exposure time varied from 5 hours 42 minutes to 8 hours depending on activity of the source. Duration of exposure varied from 4 to 39 weeks. *Calibration.* Dose measurements were made with a Victoreen ionization chamber (calibrated by Nat. Bureau of Standards) placed in the center of one of the Lucite mouse cages with its aluminum top in place. The ionization chamber was covered with a close fitting 4 mm Lucite cap of sufficient thickness to provide electronic equilibrium for cobalt-60 γ rays. Accuracy of these measurements was estimated to be ± 3%. *Challenge inoculations.* The time of each, in terms of duration of exposure, is given in Table I. Most challenge inoculations contained 60 mice from the same shipment, approximately half irradiated and half unirradiated controls. Before inoculation, the mice were redistributed to separate cage mates. The test microorganism was a streptomycin-resistant strain of *Pseudomonas aeruginosa* used for a number of years to study resistance and susceptibility in irradiated mice (1,2,5). Virulence has been maintained by weekly passage through mice. *Preparations of inocula.* An 18 hour culture was washed off an agar plate in 5 ml saline and shaken in an Erlenmeyer flask containing glass beads for 15 minutes on a rotator to break up any clumps of bacteria. The suspension was diluted to contain approximately 10^9 microorganisms/ml by means of a Coleman spectrophotometer (filter 430 m mu). Its bacterial content was checked by plating in quadruplicate 0.1 ml of the 10^{-6} and 10^{-7} dilutions. Since the LD_{100} and LD_0 of *Ps. aeruginosa* for these mice was known from past experience, inocula were chosen within these limits in an attempt to detect small differences between irradiated and control mice. Approximately 2.0, 1.2 and 1.0 x 10^8 in 0.5 ml were inoculated intraperitoneally into (usually) 10 mice each. *Leucocyte counts.*‡ At approximately weekly intervals from the 3rd to 26th week of exposure white blood counts were made on 10-20 mice in each series, a total of 492 counts, about equally distributed among the 3 series. No mouse was used more than once for this purpose.

Results. In 14 of the 23 challenge inoculations mortality in the irradiated group was practically identical with that of its control group (Table I). Slightly lower mortalities

† Operated by Univ. of Chicago for U. S. Atomic Energy Comm.

‡ Leucocyte counts were made by Betty Wolfe.

occurred among irradiated mice in 4 challenge inoculations in the 1 r series but were balanced by 4 which showed equally lower mortalities among controls. Overall, therefore, the experiments (involving 1344 mice) failed to demonstrate any consistent effect of irradiation on host resistance to experimental infection with *Ps. aeruginosa*. Nor was correlation found between duration of exposure and increased resistance or susceptibility in individual challenges. Omitted from Table I were 2 additional challenge inoculations on mice exposed 30 and 35 weeks to 0.5 r/day made with a beta hemolytic streptococcus of moderate virulence for mice (strain AD 242).§ Results with this test microorganism were inconclusive but suggestive of a very slight reduction in host resistance.

Leucocyte counts fluctuated considerably as did those reported by Lorenz *et al.*(3) and as we have found in other experiments. Their geometric means indicated occurrence of mild leucopenia (4800-5800) during prolonged exposure to 1.0 and 2.0 r/d (normal = 8500), but the leucopenia was not progressive as it was in mice exposed to 15 r/d(2). Individual counts rarely fell below 3000. Geometric means in the 0.5 r series varied from 5100 to 6400 during the 21st to 26th weeks of irradiation.

Discussion. These experiments failed to demonstrate any effect on host resistance to an experimental bacterial infection in mice exposed for many weeks to very low doses of γ radiation. The method employed, intraperitoneal inoculation with *Pseudomonas aeruginosa,* is admittedly a drastic type of challenge. It was used because it had proved satisfactory in previous experiments to demonstrate changes in host resistance of mice exposed to higher doses of X or γ radiation (1,2,5). Two trials with a β hemolytic streptococcus of medium virulence showed it to be no more satisfactory as a test microorganism.

Summary. CF-1 female mice 4 weeks of age were exposed 6 days a week to 0.5, 1 or 2 r γ radiation/day. After 4 to 39 weeks exposure they were challenged by intraperitoneal inoculation of graded doses of *Pseudomonas aeruginosa.* No detectable effect on host resistance to this experimental bacterial infection was demonstrated.

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Adrenal Cholesterol Ester Fatty Acid Composition of Different Species.* (25990)

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It is well known that adrenal tissue contains a high concentration of cholesterol of which 80 to 90% is in the esterified form(1). Administration of ACTH to rats produces a marked drop in the cholesterol ester fraction of the adrenal(2). In view of these findings, it has been suggested(3) that adrenal cholesterol esters may be involved in steroid hormone biosynthesis, particularly during stress periods when the animal is called upon to produce increased amounts of steroid hormones. Rat adrenal cholesterol esters(4) have been shown to contain a high proportion of essential fatty acids and it has been suggested(5) that those acids play a role in formation of steroid hormones. In view of the scarcity of quantitative data on distribution of fatty acids in cholesterol ester fractions of the adrenal, it was of interest to examine the cholesterol ester fatty acid (CEFA) spectrum of different species utilizing the technic of gas-liquid chromatography.

Methods and materials. All animals used in this study were males with the one exception noted in Table I. The animals were as follows: rats (Carworth strain, 250-350 g), guinea pigs (Hartley strain, 300-350 g), chickens (White Leghorn, 1-2 years), geese (mixed breeds, 1-2 years), rabbits (albino, 6 months), dogs (mongrels, 1 year and older). All animals (5-6 animals per group) were maintained on the following diets: chickens and geese—cracked corn, dogs—Purina dog chow, rabbits and guinea pigs—Purina rabbit and guinea pig chow, rats—Purina rat chow. All the animals were allowed to become adjusted to the daily routine and were not subjected to stressful conditions before being sacrificed. The animals were sacrificed and adrenals removed as quickly as possible. Human adrenals were obtained at autopsy within 24 hours after death. Adrenals of each group of animals, except the dog and man, were pooled and lipid extracts prepared as described earlier(6). Cholesterol esters were separated from the other lipid components by chromatography on silicic acid(7). The isolated cholesterol esters were interesterified in HCl-methanol and methyl esters were sublimed according to the procedure of Stoffel *et al.*(8). Gas-liquid chromatography was carried out as previously described(9).

Results. The fatty acid composition of adrenal cholesterol esters is shown in Table I. There were marked differences in distribution of the fatty acids in the cholesterol ester fraction of the adrenals of different animals. Each species appeared to possess a specific characteristic adrenal CEFA pattern. The major fatty acid of the adrenal cholesterol ester fraction of the dog, man, goose, chicken and rabbit was oleic acid. In the case of the goose this acid accounted for 79.6% of total CEFA; the rat had the lowest amount of that acid (21.1%). Each of the species studied had appreciable amounts of palmitic acid (from 7.8 to 25%). There were also differences in proportion of the other saturated and monoenoic acids characteristic of the different animals. Adrenal CEFA composition of the rat is noteworthy since it has distinctively more polyunsaturated fatty acids than any of the other animals. A large proportion of CEFA of rat adrenal was arachidonic acid (23.1%). It was also observed that a fatty acid with a retention volume equivalent to a C_{22} tetraenoic acid was present in substantial amounts (13.4%). This acid was also detected in the adrenal CEFA of man and dog. Acids with more than one double bond constituted up to 48.1% of total fatty acids in the rat. The dog was the only other animal that had appreciable amounts of polyunsaturated fatty acids (32.4%). That animal also

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TABLE I. Fatty Acid Composition of Adrenal Cholesterol Esters in Different Species.

Fatty acid†		Cholesterol ester						
Chain length carbons	No. double bonds	Rat (24)‡	Dog (5)	Man (3)§	Goose (6)	Chicken (6)	Rabbit (6)	Guinea pig (6)
		% total fatty acids						
6 to 12		1.6 ± .4*	1.7 ± .8	.9	1.0	.8	1.7	5.4
14	0	2.4 ± .6	2.2 ± .9	3.9	.3	1.2	2.9	6.2
"	1	1.2 ± .4	.5 ± .5	.6	tr	.3	1.0	1.4
16	0	17.3 ±3.0	11.0 ±3.2	15.4	7.8	12.6	17.6	25.0
"	1	2.7 ±1.7	5.7 ±1.2	6.8	2.0	8.6	6.2	10.6
"	2	.2 ± .0	tr	.2	tr	tr	tr	.9
18	0	4.3 ± .5	7.5 ±1.6	7.7	1.9	3.3	3.4	3.1
"	1	21.1 ±1.6	36.7 ±6.2	47.1	79.6	63.1	43.4	24.4
"	2	7.4 ± .9	17.0 ±3.6	2.9	4.8	5.2	11.4	9.4
"	3	2.5 ± .7	.7 ± .4	1.0	.2	.8	.9	tr
"	4	tr	tr	1.8	tr	.8	tr	"
20	0	1.3 ± .5	"	tr	"	.3	1.1	6.0
"	3	1.5 ± .6	5.5 ±1.7	2.7	"	1.2	2.1	tr
"	4	23.1 ±1.9	9.2 ±2.0	5.1	2.4	1.8	8.3	7.6
22‖	4	13.4 ±3.0	2.1 ± .9	3.9			tr	

* Stand. dev.

† Represents major acids found; small amounts of others were also detected.

‡ Represents No. of animals; values on rat represent avg of 4 pools of 6 rats, and on man and dog individual analysis. All animals were males except 3 geese.

§ Insufficient values for reliable stand. dev.

‖ Identification based on retention time.

had the highest concentration of C_{20} trienoic acid.

Discussion. The present study shows that each species has a characteristic adrenal CEFA spectrum. The differences between the CEFA in the adrenal of the different species do not appear to be related to diet. Diets consumed by the animals were previously analyzed for fatty acid composition(10). All of the diets were low in fat (2 to 4%), with the exception of man, but dietary fat was high in linoleic acid (30 to 54%). All species had a smaller proportion of linoleic (4.8 to 17.0%) and some species (dog, man, goose, chicken and rabbit) had a higher proportion of oleic acid in their adrenal CEFA than was present in the diet. The fatty acid spectrum of the adrenal cholesterol esters does not bear a close similarity to that of the serum cholesterol esters in each species. In most of the species studied(10) the major fatty acid of the serum cholesterol ester fraction was linoleic acid and in the adrenal fraction oleic acid is the major acid. The only similarity which does exist relates to the fact that those species which have large amounts of polyunsaturated fatty acids (rat and dog) or oleic acid (chicken and goose) in adrenal CEFA also have large amounts of those acids in serum CEFA. Blood cholesterol esters of the rat, human and dog were checked for C_{22} tetraenoic acid and only traces could be detected.†

The significance of the adrenal polyunsaturated fatty acid which may be a C_{22} tetraenoic acid is not apparent. Further studies are underway to characterize that acid more completely. Whether specific cholesterol esters play an important role in biosynthesis of steroid hormones is not known. Preliminary experiments have indicated that there may be changes in proportion of arachidonic acid and the C_{22} tetraenoic acid of the adrenal cholesterol esters following administration of ACTH to rats.†

Summary. The fatty acid composition of adrenal cholesterol ester fraction has been determined by gas-liquid chromatography in 7 species. Characteristic CEFA patterns for each type of animal were apparent. Rat and dog had the highest content of polyunsaturated fatty acids in the adrenal cholesterol ester fraction. The remaining species had considerably less of those acids and the major CEFA of dog, man, goose, chicken and rabbit

† Unpublished observations.

was oleic acid. In the dog, rat and human, an acid tentatively identified as a C_{22} tetraenoic acid was found in the adrenal CEFA. The significance of these results in relation to hormone synthesis and polyunsaturated fatty acid metabolism is discussed.

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Plasma Disappearance Rate and Tissue Distribution of Radioactive Cobalt Labelled Cyanocobalamin Injected into Various Animals.* (25991)

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In humans and laboratory animals, parenterally injected cyanocobalamin is rapidly cleared from the blood stream and approaches normal levels within 24-48 hours as determined by microbiological assay methods(1-3). With increased sensitivity attainable with cobalt-60 labelled cyanocobalamin, Miller *et al.* (4) demonstrated that an injected dose of the radioactive vitamin disappeared from the plasma more slowly in patients with chronic myelogenous leukemia than from the plasma of normal subjects. After 24 hours, sufficient radioactivity remained in the plasma to determine the slope of the exponential disappearance curve, which approximated a half life of 5 days. In female rabbits, Rosenthal (5) found that plasma disappearance curves beginning 48 hours after injection of cobalt-60 cyanocobalamin could be resolved into 2 components with apparent half lives of 4.1 days (Component A) and about 50 days (Component B) respectively. The similar plasma disappearance rate of cyanocobalamin in human subjects and rabbits prompted us to determine plasma disappearance rate of injected radio cyanocobalamin in a variety of animal species.

Materials and methods. White rabbits and white leghorn chickens weighing 2-3 kg were obtained from commercial sources. Mongrel dogs weighing 6 to 15 kg were obtained from the local pound. Healthy male human subjects were laboratory personnel weighing 64 to 86 kg. All laboratory animals were maintained on commercial animal feed; water was available *ad lib.* The human subjects ate their usual diets without restriction. The animals were injected with Co^{60}-cyanocobalamin intramuscularly into the left hind leg or intravenously *via* the ear vein in rabbits, wing vein in chickens and jugular vein in dogs. The human subjects were injected with Co^{58}-cyanocobalamin *via* the antecubital vein.

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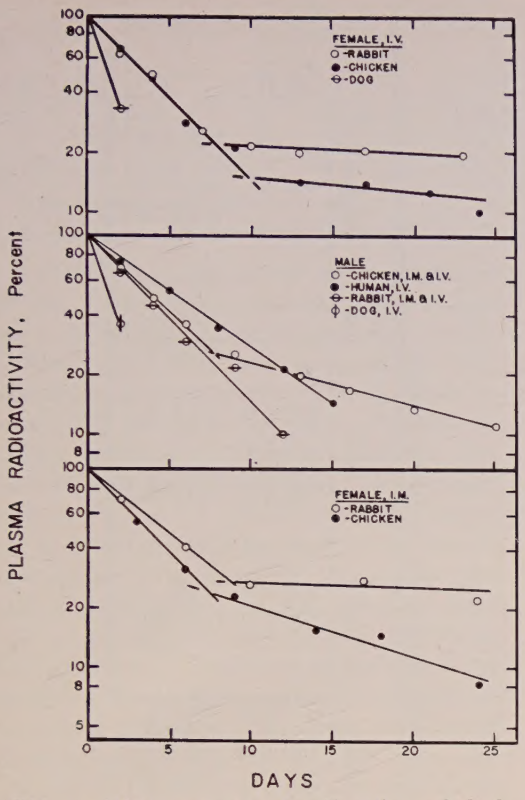

FIG. 1. Disappearance of radioactive cobalt labelled cyanocobalamin from plasma of various animals. Initial values at zero time represent residual radioactivity present in plasma 2 days following inj. of cyanocobalamin. The lines were fitted to experimental values by method of least squares. Each point represents avg values obtained on 3 to 6 animals.

Two days after administration of radioactive vitamin, 8 to 10 ml of blood was obtained for initial estimation of radioactivity by heart puncture from rabbits and chickens, from the jugular vein of dogs and antecubital vein of the human subjects. Similar samples were obtained at periodic intervals. At termination of experiments the rabbits, dogs and chickens were sacrificed and the tissues analyzed for radioactivity by methods previously described (5). Radioactivity in 5 ml samples of plasma was counted in a well type scintillation counter and compared with a standard prepared from the original material at time of injection. Plasma samples were obtained for analysis of radioactivity until the counting rate approached 1.2 times background where the standard error of the count became greater than $\pm$ 10% for a minimum of 10,000 counts. This approximated 3 x 10^{-5} μc per 5 ml of plasma.

Results. The plasma disappearance curves of radio-cyanocobalamin (Fig. 1) were of 2 types, a single component curve in male human subjects and rabbits and a 2 component curve in chickens and female rabbits. The most rapidly disappearing component is designated Component A and the more slowly disappearing one as Component B.

In dogs, plasma radioactivity reached negligible levels within 4 days after injection. Urinary excretion of radioactivity was determined in some dogs and represented less than 2% of injected dose. This value is less than

TABLE I. Plasma Biological Half Life of Radioactive Cyanocobalamin Injected into Various Animals.

Species	Sex	Route	Dose, μc/kg*	Half life $\pm$ S.E. (days) Component A	Component B
Chicken	♀	Intramusc.	.104	4.21 $\pm$ .42 (6)	9.75 $\pm$ 1.01 (6)
	♀	Intrav.	.121	4.06 $\pm$ .34 (6)	31.6 $\pm$ 13.2 (6)
	♂	Intramusc.	.122	4.10 $\pm$ .22 (3)	11.6 $\pm$ .82 (3)
	♂	Intrav.	.140	4.08 $\pm$ .45 (4)	12.5 $\pm$ 1.37 (3)
Rabbit	♀	Intramusc.	.056	4.68 $\pm$ .32 (5)	89.8 $\pm$ 64.5 (3)
	♀	Intrav.	.050	4.34 $\pm$ .29 (6)	38.1 $\pm$ 16.3 (5)
	♂	Intramusc.	.044	3.91 $\pm$ .13 (4)	N.D.
	♂	Intrav.	.046	3.82 $\pm$ .53 (4)	"
	Weighted avg			4.18 $\pm$ .13	29.9 $\pm$ 9.3
Human	♂	Intrav.	.058	5.68 $\pm$ .45 (4)	N.D.
Dog	♀	"	.085	1.34 $\pm$ .27 (4)	"
	♂	"	.069	1.82 $\pm$ .81 (4)	"

* Specific activity of Co^{60} and Co^{58} labelled cyanocobalamin was approximately 1 μc/μg.
No. of animals in parentheses. N.D. = not detected.

urinary excretion of 4% of injected dose previously found in rabbits under similar conditions(5).

The individual disappearance curves were fitted with straight lines by the method of least squares and half life of each component was calculated (Table I). The half life of component A for chickens and rabbits of both sexes was remarkably similar and averaged 4.18 days. The half life of component B, averaging 29.9 days, was significantly longer than that of component A. In male human subjects the half life of component A was 5.68 days. In dogs of both sexes the value was less than 2 days, but this value is of doubtful reliability due to rapid disappearance of the vitamin from the blood stream.

Distribution of radioactivity in tissues of rabbits and chickens 26 days following injection of cobalt-60 cyanocobalamin are shown in Table II. In rabbits, liver and kidney contained the greatest amount in approximately equal quantity, muscle and brain tissue the smallest amount, and heart tissue an intermediate quantity. Distribution of tissue radioactivity appears to be independent of sex or of route of administration of injected vitamin.

In chickens, relative distribution of radioactivity between various tissues was similar to rabbits. Of special interest is the smaller amount of activity present in light meat as compared to dark meat and the similarity between heart and gizzard activity. Specific activity of the liver, kidney, heart and spleen of chickens was usually several times higher than that found in comparable rabbit tissues, but chicken muscle contained less than rabbit muscle.

Tissue content was generally lower in dogs than in rabbits sacrificed 7 days after dosage but relative concentration between tissues was similar for both species with the exception of muscle tissue (Table III). Dog muscle contained approximately 15% of that found in rabbits while other tissues contained about 50%. As with rabbits and chickens, little, if any, differences existed in radioactive content of tissues obtained from male or female dogs.

Discussion. Previous studies have shown

TABLE II. Tissue Radioactivity 26 Days Following Injection of Co^{60} Cyanocobalamin.

Species	Sex	Route	No. animals	Liver	Kidney	Heart	Spleen	Muscle Dark	Muscle Light	Gizzard	Brain
Rabbit	♂	Intrav.	4	10.67 ± 1.55	11.85 ± 2.77	4.04 ± .25	3.78 ± .31	1.78 ± .23			2.19 ± .56
	♂	Intramusc.	4	12.19 ± 2.15	15.05 ± 3.99	4.85 ± 1.32	4.31 ± 1.74	3.69 ± .53			2.82 ± .83
	* ♀	Intrav.	5	13.75 ± .59	15.36 ± 1.45	6.73 ± .80	4.41 ± 1.10	2.40 ± .24			2.61 ± .77
	* ♀	Intramusc.	6	13.52 ± 1.70	15.58 ± 2.99	5.79 ± .83	4.82 ± 1.36	2.79 ± .32			
Chicken	♂	Intrav.	3	56.47 ± 9.02	64.63 ±10.50	6.38 ± 4.08	9.49 ± .57	1.26 ± .08	.42 ± .04	5.19 ± .54	
	♂	Intramusc.	4	46.04 ± 3.18	67.42 ±14.24	12.69 ± 1.93	11.32 ± .44	1.36 ± .20	.63 ± .21	4.08 ± .66	
	♀	Intrav.	3	84.15 ± 5.59	86.67 ±26.81	22.82 ± 1.97	18.62 ± 2.82	1.64 ± .17	.52 ± .09	6.99 ± 2.70	
	♀	Intramusc.	4	82.08 ± 7.64	68.39 ±10.90	17.85 ± 1.05	14.56 ± 1.88	1.39 ± .19	.86 ± .09	6.34 ± 1.12	

* From Rosenthal, 1959(5).

Values in terms of percentage of dose/100 g tissue ± stand. error.

TABLE III. Tissue Radioactivity 7 Days Following Injection of Co^{60} Cyanocobalamin.

Species	Sex	Route	No. animals	Liver	Kidney	Heart	Spleen	Muscle	Brain
Dog	♂	Intrav.	4	4.13 ± .41	5.32 ± .48	2.32 ±.62	6.42 ± .22	.44 ±.09	1.19 ±.22
	♀	"	4	5.96 ± .84	8.25 ± .36	3.85 ±.79	8.77 ±1.88	.59 ±.08	1.61 ±.13
Rabbit*	♀	Intramusc.	6	13.28 ±1.16	12.67 ±1.62	7.16 ±.27	10.80 ±1.99	3.56 ±.11	2.80 ±.59

* From Rosenthal, 1959(5).

Values in terms of percentage of dose/100 g of tissue ± stand. error.

that plasma level and tissue concentration of Vit. B_{12} varies widely between different species of animals(6-8). It is somewhat surprising therefore, to find that the apparent disappearance rate of component A in plasma is so remarkably similar in animals as diverse as rabbits, chickens and human subjects. The different half life of component A for human subjects (5.7 days) as compared with chickens and rabbits (4.2 days) may be due to experimental error since, in the smaller animals, withdrawal of sufficient blood to yield 5 ml of plasma for radioactivity determinations represents a significant proportion of the blood volume with concomitant removal of the bound vitamin. This tends to decrease the value of the observed half life. In contrast to the laboratory animals, withdrawal of similar quantities of blood from human subjects represents a small percentage of blood volume. If one estimates a correction for removal of radioactivity, the half life of component A for chickens and rabbits becomes 5.3 days, a value consistent with that for male human subjects. The 5.7 day half life for component A in normal human subjects reported here is surprisingly similar to the 5 day half life found in sera of patients with chronic myelogenous leukemia determined by Miller *et al.*(4). Furthermore, Reizenstein(9) has recently calculated the apparent biological half life of plasma Vit. B_{12} in human subjects to be 6 days, a value in complete agreement with our experimental data.

The finding that 2 well defined disappearance curves are present in plasma of female rabbits and only one in male rabbits, suggests the possibility of an endocrine relationship. On the other hand, no sex relationship is apparent in chickens.

Disappearance rates shown in Fig. 1 and Table I have not been corrected for disappearance rate of component B. It must be recognized that disappearance rate of component A may be modified by the slower disappearance rate of component B. Until further information is available, it appears desirable to present our data in its actual uncorrected form.

Although Vit. B_{12} content of serum varies widely between various species of animals, the similarity of disappearance rate of component A indicates that the protein-cyanocobalamin complex in various animals may be qualitatively similar. On the other hand, Rosenthal(10) showed that disappearance rate of component A in rabbits given oral doses of Co^{60} cyanocobalamin was slower ($T\ ½ = 7.1$ days) than parenterally injected vitamin. These data demonstrate qualitative differences in binding affinities depending on route of administration.

The wide variation of radioactivity in tissues of various species demonstrates that comparable tissues from different animals have different Vit. B_{12} binding affinities. This is evident by comparing tissues of chickens and dogs with that of rabbits. Reasons for this wide variation are unknown and must await further clarification.

Summary. Plasma disappearance of parenterally injected radio cyanocobalamin in various animals was determined. Beginning 48 hours after injection 2 disappearance curves were found in female rabbits and chickens of both sexes, but only 1 disappearance curve was present in male rabbits and male human subjects. The most rapidly disappearing component (Component A) was similar in rabbits and chickens ($T\ ½ = 4.2$ days) and human subjects ($T ½ = 5.7$ days). The more slowly disappearing component (Component B) in female rabbits and chick-

ens of both sexes approximated a biological half life of 29.9 days. In dogs of both sexes, plasma disappearance rate was very rapid (T ½ = 1.5 days). Tissue radioactivity varied between tissues of the same species and between the various species studied.

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Isolation and Elimination of Pleuropneumonia-Like Organisms From Mammalian Cell Cultures.* (25992)

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Following the report of Robinson, *et al.*(1) on association of pleuropneumonia-like organisms (PPLO) with human cells in culture, high incidence of PPLO contamination has been found in several laboratories(2-4). Our findings have been briefly presented in preliminary communications(5,6). Methods for surveillance of animal cell cultures for PPLO and for their elimination are important to application of cell cultures in virological and physiological research, since PPLO contamination is not revealed by grossly visible changes in culture appearance or cellular morphology. Neither are influences of PPLO on metabolism of animal cells in culture known. This paper reports results of a program instituted in the Dept. of Bacteriology, Univ. of Minnesota, for isolation and cultivation of PPLO from established animal cell lines. Use of the antibiotic kanamycin for elimination of PPLO from contaminated cell lines is described.

Materials and methods. Cells tested were mostly those propagated in this laboratory in continuous culture for varying periods from 2 to 5 years. Twenty-four lines submitted by other investigators were also tested. Since results were communicated individually to submitting investigators, these results are combined with other data for presentation here. Fifteen lines, all but one propagated by the same worker for more than 2 years, were used for evaluation of the effectiveness of kanamycin. Cell lines from Minnesota were propagated as described(7), while other cells were cultivated in routinely employed media. All media contained human, horse, calf or rabbit serum supplemented with a) yeast extract in Hanks' balanced salt solution or b) Eagle basal medium(8). Serum pools were Selas-filtered and inactivated at 56°C for 30 minutes before use. *Trypsin* (Difco 1:250) in 10 times stock solution was prepared by dissolving 0.2 g in 100 ml of GKN solution (5.5 mM glucose, 5.4 mM potassium chloride, 138 mM sodium chloride). Trypsin stock was adjusted to pH 7.8 with sodium bicarbonate (166 mM) solution, sterilized by Selas filtration, and stored at –20°C. *Kanamycin sulfate*† was dissolved in Hanks' balanced salt solution to a concentration of 10^4 μg kanamycin base per ml.

PPLO medium, modified from the formulae

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† Kanamycin sulfate was supplied kindly by Dr. C. H. Mann, Bristol Labs., Inc.

of Brown(9) and of Robinson and Wichelhausen(10), was prepared by mixing 250 g ground beef heart with 900 ml of distilled water and 5 g sodium chloride. Pancreatin (Baltimore Biol. Labs., lactose-free), 2.5 g, was dissolved in 100 ml of 0.5% sodium chloride and added to the beef-water mixture after it was pre-warmed in a water bath to 50-56°C. After digestion for 2 hours with frequent agitation the mixture was filtered through 4 layers of gauze, and the filtrate boiled for 5 minutes and filtered through coarse filter paper. The solution was heated to boiling, restored to volume and filtered through Whatman No. 2 paper. The broth was enriched with 1 g yeast extract (Difco) per liter, cooled, adjusted to pH 8 with 1 N sodium hydroxide solution, and dispensed in amounts suitable for autoclaving at 121°C for 15 minutes. For solid medium, 1.5% Difco special agar (Noble) was added to the broth and dissolved by boiling. After autoclaving and before use, both liquid and solid media were enriched with 20% filtered human ascitic fluid previously tested for capacity to support growth of PPLO.

Results. Isolation of PPLO from cell lines in continuous culture. Cultures of 166 sublines of 68 cell lines were tested for PPLO contamination over a period of 18 months. Cultures were propagated in 1 ml of medium in screw-cap tubes, and were fed twice weekly by complete medium replacement. Cell suspensions prepared for passage of cell cultures, fed 24 hours previously, were employed for isolation of PPLO. Monolayers were rinsed with GKN and dispersed in 1 ml of 0.02% trypsin allowed to act for 1-2 minutes. After dispersion by pipetting, cells were sedimented at 40-50 x gravity for 3-5 minutes. The supernate was discarded and the cells were suspended in conditioned medium containing sufficient fresh growth medium for establishment of new cultures. From 1 ml of this suspension, 0.1 ml was immediately inoculated into 5 ml of Difco trypticase soy broth containing 0.1% agar, 0.02 ml streaked on an agar plate of the PPLO test medium, and the remainder inoculated into PPLO broth medium. After 24 hours at 37°C PPLO broth and agar subcultures were prepared from the inoculated tube of PPLO broth. After 24 hours more incubation, the first and second PPLO broth cultures were inoculated on agar medium. Plates were examined with a stereoscopic microscope daily for the first 4 days and every day thereafter for a period of 2 weeks. Trypticase soy broth cultures were examined routinely for turbidity as an indication of ordinary bacterial contamination. Data are given in Table I. Results of repeated tests of given cell sublines were consistently negative or positive for presence of PPLO, with the exception of variable results observed with 5 sublines. PPLO isolated from 2 of the sublines listed in Table I, clonal HeLa 4 and altered monkey kidney(11), grew sparsely and slowly. With most but not all cell lines, at least one subline was found uncontaminated to serve for regeneration of stock; for decontamination of the remainder, an antibacterial agent effective against PPLO but nontoxic for cells was desirable.

Elimination of PPLO contamination with kanamycin. Preliminary survey of antibiotics and chemotherapeutic agents showed that most of the broad-spectrum antibiotics were effective against PPLO in high concentration. Only kanamycin(12) could be used in relatively high concentration without detrimental effect on cell morphology and growth. Fifteen PPLO-contaminated cell sublines were used to study the value of kanamycin for decontamination. Six replicate cultures per line were established by incubation for 3 days. Medium was replaced completely with fresh growth medium; 3 of the tube cultures received 100 μg of kanamycin in the 1 ml of replacement growth medium. Twice during the following treatment period, 1 control and 1 treated culture from each line were redispersed with trypsin and used for propagation of 3 new cultures each. Three days after the second such passage, kanamycin treatment was discontinued and both control and treated lines maintained independently thereafter in identical media. Total period of treatment was 3 weeks. Cells were tested for PPLO contamination 12 days after discontinuation of treatment, and retested at approximately monthly intervals thereafter. Results listed in Table II show that in no case was a treated

TABLE I. Summary of Results of Cultures for PPLO from 166 Sublines of Mammalian Cells.

Source	Total sublines studied	Negative		Positive		Variable		
		No. sublines	Total cultures	No. sublines	Total cultures	Subline	Cultures Pos	Neg
Human	110	45	207	63	258	Minn. HeLa E	13	2
						Clonal Minn. HeLa 4	8	6
Rabbit	39	12	32	25	31	Minn. 58-3-1 (E)	2	1
						Minn. 58-5-3 (E)	1	1
Monkey	5	3	15	1	2	Altered monkey kidney	7	4
Mouse	2	2	6					
Porcine	5	5	17					
Bovine	2	2	12					
Misc.	3	3	14					
Total	166	72	303	89	291	5	31	14

culture found positive. Control cultures continued to yield PPLO consistently with one exception, the clonal Minnesota HeLa, which carries a strain of PPLO that grows slowly and is difficult to isolate. The PPLO-free cell sublines obtained by this kanamycin treatment have remained free of PPLO for over 10 months. Kanamycin has since been employed successfully for elimination of PPLO from other cell lines. Since the studied lines represent a heterogeneous group obtained from various sources and cultivated in different media, the findings suggest that kanamycin treatment may be broadly useful for elimination of PPLO from contaminated animal cell cultures. PPLO isolated from untreated contaminated cell lines are shown in Fig. 1; a spectrum of morphological colonial types is represented.

Intensity of kanamycin treatment for PPLO elimination. A series of tube cultures, initiated with 25,000 cells per ml culture medium, of a clonal subline of the Minnesota cell line 55-12-1 of human esophageal epithelial

TABLE II. Results of PPLO Cultures from 15 Kanamycin-Treated* Mammalian Cell Lines and Parallel Controls.

Cell line	Medium†	Ref	Control‡		Treated‡	
			Pos	Neg	Pos	Neg
Horse HeLa 100	A	13	10	0	0	10
Idem	B		10	0	0	10
MBA HeLa	B	14	10	0	0	10
HeLa E	C		12	0	0	10
" KC	C		9	0	0	9
Clonal HeLa 4	C		7	4	0	9
" EE (Minn.)	C		10	0	0	10
Chang conjunctiva	A	15	10	0	0	10
Clonal Chang conj. B_2a_2	C		4	0	0	4§
" " " B_2a_4	C		10	0	0	10
Lass liver (Minn.)	C	16	10	0	0	10
Clonal Chang liver A_1a_1	C	15	10	0	0	10
" " " D_1b_1	C		9	0	0	9
" " " A_2b	C		9	0	0	10
U-12	A	17	9	0	0	9

* Treatment begun 6/26/59 and discontinued 7/17/59.

† Medium: A = HoS–20, YEM–80; B = HoS–40, BSS–60; C = HuS–20, YEM–80.
HoS = horse serum, HuS = human serum, BSS = Hanks' solution, YEM = 0.1% yeast extract in BSS with 2.5 mM $NaHCO_3$ when used with HuS or 4.2 mM $NaHCO_3$ when used with HoS.

‡ Cultures performed monthly July 1959 to June 1960.

§ Kanamycin-treated culture lost through contamination after 10/7/59.

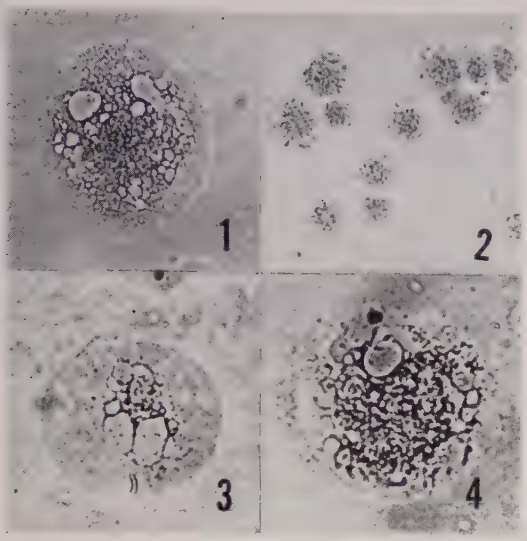

FIG. 1. Colonies of PPLO isolated from mammalian cell cultures. 1. Minnesota HeLa. 2. Clonal Chang liver A_1a_1. 3. Horse HeLa 100. 4. Clonal Chang conjunctiva B_2a_4. × 210.

cells (EE) was treated with kanamycin in graded concentration. At intervals, cultures were rinsed 3 times with balanced salt solution to remove antibiotic; cells were grown for 1 week in antibiotic-free medium, and cultures were trypsin-dispersed for detection of PPLO. Results are tabulated in Table III. It is evident that the time of kanamycin treatment for elimination of PPLO from this cell line could be shortened considerably depending on antibiotic concentration. In contrast to the 3-week treatment arbitrarily used in the first study, 48 hours exposure to 100 μg per ml of kanamycin appeared to present an adequate treatment period with a 4-fold safety margin. Repetition of this experiment gave similar results, as did a similar experiment with the Minn. 56-6-2 line (Lass) of human liver cells(16).

Discussion. Our experience with the described medium and technic for isolation of pleuropneumonia-like organisms from animal cell cultures suggests consistent sensitivity. Although we were concerned mainly with detection of PPLO contamination in our own cell lines and repropagation of uncontaminated lines rather than comparative evaluation of various media and technics, it may be helpful to report that an agar medium commonly employed for isolation of PPLO was not found sufficiently adequate, and that freshly trypsinized cells from monolayer cultures suspended in their growth medium were superior material for consistent isolation of PPLO. Our findings that 65 sublines of human cells in continuous culture were contaminated, as well as 29 sublines of other species. reinforces the experience of other laboratories that PPLO contamination is a widespread danger in cell culture studies. Circumstantial as well as serological(18) evidence indicates that PPLO strains of human origin are a major source of this contamination. Although a high proportion of our cell lines were contaminated with PPLO, evidently a number of lines had been maintained uncontaminated for years. Variation in incidence of PPLO contamination of cell lines maintained in different workrooms of our laboratory was suggestive but did not provide definitive evidence for particular routes of entry of con-

TABLE III. Recovery of PPLO from Contaminated Minn. EE Cell Cultures Treated Variably with Kanamycin.

Medium conc. of kanamycin, μg/ml	Hr of treatment* .5	1	2	3	4	6	10	24	36	48	102
1,000.0	+†	—	—	—	—	—	—	—	—	—	—
320.0	+	+	—	nd	+	—	-	—	-	-	—
100.0	+	+	+	+	—	+	—	-	-	-	—
32.0	+	+	+	+	+	+	—	—	—	—	—
10.0	+	+	+	+	+	+	+	V	V	V	V
3.2	+	+	+	+	+	+	+	+	+	+	+
1.0	+	+	+	+	+	+	+	+	+	+	+

* Replicate monolayer tube cultures exposed to kanamycin in culture medium; after exposure, cultures washed 3 times with BSS, grown 1 wk on growth medium without antibiotic, and dispensed for passage and culture for PPLO.

† + = isolation of PPLO at first and second culture passage after kanamycin treatment; — = failure to isolate PPLO in 2 attempts; V = positive results for one attempt and negative results for others; nd = not done.

taminants into cultures. It was encouraging to find that adequate surveillance was possible by use of the pancreatic digest medium for PPLO isolation, and that decontamination could be accomplished with an antibiotic well tolerated by animal cells. If the efficacy of brief kanamycin treatment is confirmed by continued study of treated cell lines, decontamination with kanamycin will be additionally attractive. Such brief treatment is desirable to permit evaluation of any selective effects of kanamycin on culture evolution, not reflected in visible morphologic influence. Treatment with relatively high levels of kanamycin also is desirable to prevent emergence of antibiotic-resistant PPLO strains. The data of Smith *et al.*(19) indicate that at least 1 human cell line, the Eagle strain KB, is not inhibited in growth by kanamycin in concentration less than 500 μg per ml; the 100 μg per ml level employed in our studies therefore may be well within safe limits for cells. It should be emphasized that the cultures studied here were propagated routinely in medium not containing penicillin or streptomycin. If isolated PPLO are in reality L forms induced by prior use of these antibiotics during culture propagation, then maintenance of kanamycin-treated cell lines without recontamination is understandable. If, alternatively, the PPLO are organisms of human origin as suggested by serological evidence from other laboratories, then successful maintenance following kanamycin treatment of cultures indicates that PPLO contamination can be avoided by rigorous aseptic cell-cultural technic. Prolonged propagation of treated cultures without antibiotic suggests that properly prepared serum is not a likely source of contamination. Although we have not yet encountered kanamycin-resistant PPLO in cell cultures, the possibility suggests that kanamycin should not be regarded as a prophylactic substitute for adequate preventive technic, but only as a promising therapeutic agent for rescue of valuable contaminated cell lines.

Summary. One hundred sixty-six sublines representative of 68 culture lines of cells of human, rabbit, monkey, mouse, pig, cow and other species origin were tested repeatedly for contamination with pleuropneumonia-like organisms over a period of 18 months. Cells freshly dispersed with trypsin were inoculated into a medium containing pancreatic digest of beef heart enriched with yeast extract and human ascitic fluid. Of the tested sublines, 57% were contaminated with PPLO. Repeated tests with the heart digest medium on 89 sublines were consistently positive, while 5 sublines yielded variable results. PPLO strains isolated from 2 of the variable cell sublines grew sparsely and slowly, but growth of other PPLO strains was luxuriant in the test medium. Fifteen cell lines carrying PPLO of varying colonial morphology were treated for 3 weeks with 100 μg of kanamycin per ml of medium. Treated sublines yielded no PPLO during subsequent cultivation for nearly a year in the absence of kanamycin. A contaminated strain of human esophageal epithelium has been freed of PPLO by brief exposure to kanamycin. Required time of exposure appeared inversely related to kanamycin concentration employed.

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Effect of X-Radiation on Uptake of P^{32} into Individual Nucleotides of HeLa Cells.* (25993)

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Separation of the acid soluble phosphorus compounds of transplanted mouse mammary carcinoma and determination of their relative specific activities 2 hours after injection of radioactive phosphate have been described (1). It was demonstrated that 4500 r (in air) localized to the tumor brought about a lowering of relative specific activities of some polyphosphonucleotides. Because these tumors contained a variety of cells, it was thought that the results obtained might be due to changes in metabolism of cells other than those of the mammary carcinoma. To investigate the effect of non-malignant cells, the work was repeated employing Ehrlich ascites carcinoma grown in peritoneal cavities of susceptible mice. These cells can be isolated as a relatively homogenous line. The results of this study are being described elsewhere(2). As a further step in employment of a pure cell line, we have continued our studies using HeLa cells grown and irradiated *in vitro*. This latter investigation is the subject of this report.

Materials and methods. Cultures of HeLa cells(3) were propagated in Eagle's basal medium(4) supplemented with 10% human serum. Cells were harvested by rinsing with bicarbonate buffered glucose-KCl-NaCl solution followed by dispersal with 0.05% trypsin (Difco 1:250) as previously described(5). The trypsinized cells from about 100 bottles were pooled and centrifuged at 200 g for 10 minutes. Cells were resuspended in 25 ml of 10% human serum, 90% Hanks' balanced salt solution and enumerated. The suspension was divided and one-half containing at least 10^8 cells was subjected to irradiation. The bottle containing the cells was placed on its side on pressed wood backing material. The surface of the medium was 35 cm from the focal spot of the x-ray tube. The x-ray machine was operated at 220 KP, 15 MA with no filter (HVL 0.35 mm Cu). A total dose of 5000 r including backscatter was delivered at surface of medium. Cells were then incubated for 2 hours at 37°C, 4 ml of a solution of disodium hydrogen phosphate containing 200 μc of P^{32} having been added to each 30 ml of suspension. After centrifugation 200 g for 10 minutes, supernate was discarded. The cells were resuspended in 20 ml of Hanks' balanced salt solution and recentrifuged. The washed cells were homogenized with 4 volumes of ice cold 0.6 N perchloric acid in a glass Potter-Elvehjem homogenizer. After refrigeration for 30 minutes at 4°C, the homogenate was centrifuged in the cold at 1300 g for 30 minutes. Supernate was decanted off, and precipitate washed with 5 ml of 0.2 N perchloric acid. The combined extract and the washings were neutralized with cold 5 N potassium hydroxide and refrigerated overnight. After removal of potassium perchlorate by centrifugation, aliquots were taken for determination of radioactivity, total phosphorus(6), and nitrogen(7). The neutralized extracts were placed on twin columns of Dowex 1 (formate) X 8, 200-400

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mesh and eluted by gradient elution with formic acid and ammonium formate buffers as previously described(1). For preparations representing 2 x 10^9 cells, a column size of 33 cm x 2.4 cm was used. Volumes of eluants used were as follows: water, 1 bed vol.; 0.2 M formic acid, 2 vol.; 0.5 M formic acid, 1 vol.; 1 M formic acid, 1 vol.; 2 M formic acid, 5 vol.; 4 M formic acid, 2 vol.; 0.2 M ammonium formate in 4 M formic acid, 6 vol.; 0.4 M ammonium formate in 4 M formic acid, 2 vol.; 0.8 M ammonium formate in 4 M formic acid, 4 vol. Rate of flow was 5-6 ml in 25 minutes. An automatic scanning device was used to monitor radioactivity of the fractions eluted. Those showing radioactivity were carefully counted and their extinctions at 260 mμ determined. Values obtained were plotted against volume of eluate. Fig. 1 shows graphs obtained with 2 columns on which identical extracts were fractionated and demonstrates the reproducibility of the method. Fig. 2 shows the fractionation of extracts of (A) non-irradiated cells, (B) irradiated cells. Fractions forming each peak were pooled and a number of aliquots used for determination of specific activity of the phosphorus compound. Since inorganic phosphorus was eluted along with uridylic acid and inosinic acid, specific activity of the inorganic phosphorus from each column was determined by the method of Ernster and associates(8). In Table I activity of each peak is expressed as a percentage of specific activity of the inorganic phosphorus. The remainder of each pool was lyophilized, and residue paper - chromatographed. Where amount of material was sufficient, the identity of the phosphorus compound was corroborated by determining absorption ratio 250/260 and 280/260 mμ and by rechromatographing with known controls. The protein residue remaining from extraction with perchloric acid was further extracted with boiling chloroform-methanol mixture (2:1 by volume). Specific activity of the extracted lipid material was determined.

Resutls. In the earlier experiments 21 phosphorus compounds were separated as illustrated in Fig. 1. As the technic of the column chromatography was improved it became apparent that several of these peaks could be further resolved. In Fig. 2, 24 peaks are shown. Table I gives identities of these peaks as far as we have been able to determine them. Relative specific activity (r.s.a.) is specific activity in counts per minute per μg of phosphorus as a percentage of specific activity of the inorganic phosphorus.

The neutral phosphorus fraction which passed through the Dowex 1 column formed a smaller proportion of total acid soluble phosphorus than it did in extracts from ascites cells or from mouse mammary carcinoma (1,2).

The most prominent difference between irradiated and non-irradiated cells was shown by peak XVI. The phosphorus in this peak from irradiated cells formed 9.8% of total acid soluble phosphorus while from non-irradiated it was only 4.9%. The r.s.a. was markedly changed from 0.65 in non-irradiated to 3.8 in irradiated cells.

A difference was shown in the r.s.a. of adenosine triphosphate (ATP). It was only 3.7 in irradiated as compared to 22.7 in non-irradiated cells, and total amount of ATP also was lower in irradiated cells. Amount of phosphorus isolated in the previous peak XXI was higher in irradiated cells, which might suggest there was imperfect resolution of the 2 peaks. However, it should be noted that the r.s.a. of peak XXI was the same in both irradiated and non-irradiated cells.

The r.s.a. of the total phospholipid extracted from the residue showed no difference between irradiated and non-irradiated cells.

From Table I it would appear that there were differences in r.s.a. values of peaks VI, VIII, XI and XX from irradiated and non-irradiated cells. However, the significance of these differences is questionable because of the small amount of material available for analysis. It is worthy of note that peak XIII is much more prominent in HeLa cells than in either mammary carcinoma or Ehrlich ascites cells.

Discussion. The spectrum of phosphorus containing metabolites extractable by perchloric acid from HeLa cells propagated *in*

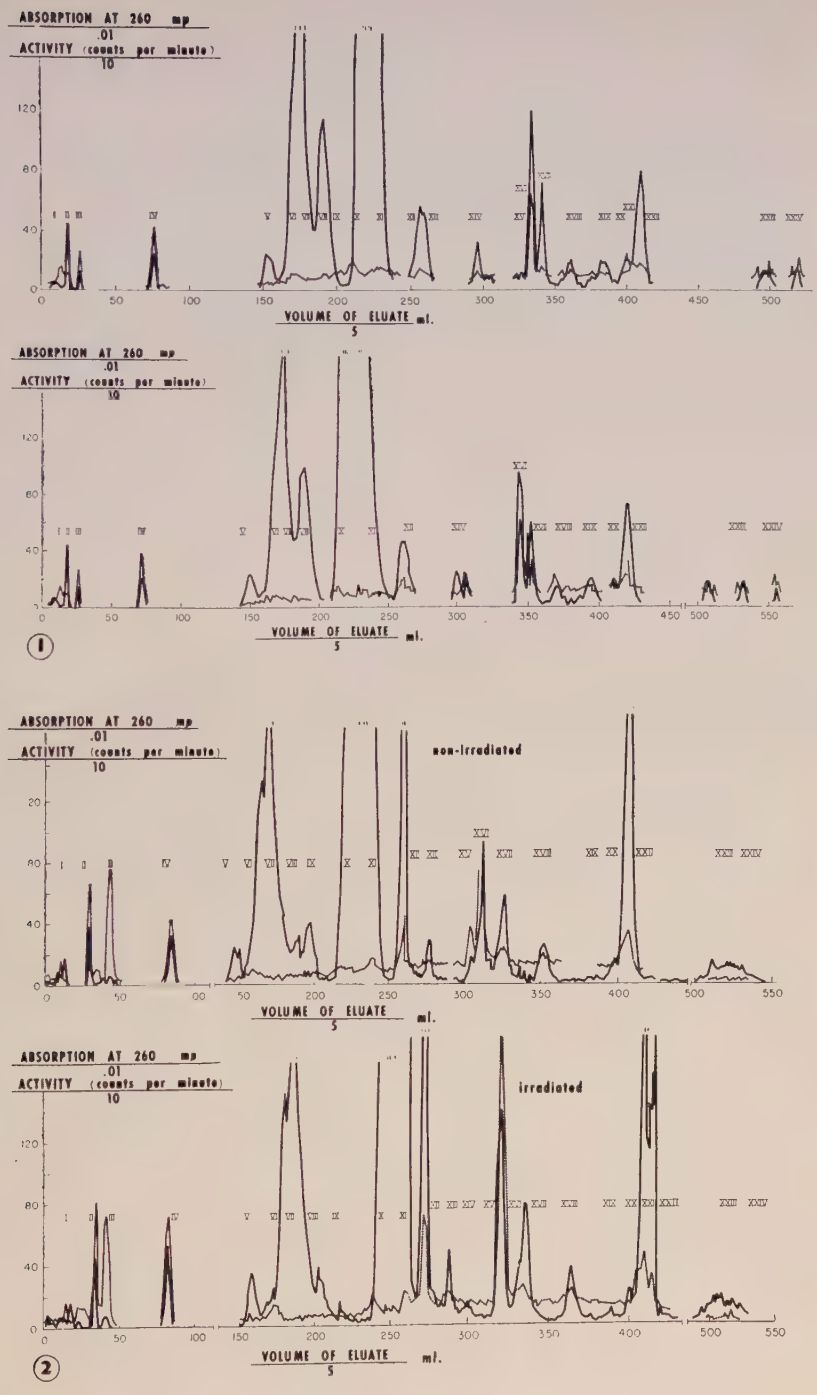

FIG. 1. Acid soluble nucleotides from HeLa cells. Upper and lower curves were obtained by elution of identical preparations from 2 columns. Dotted lines indicate absorption at 260 mμ. Solid lines indicate activity of P-32.

FIG. 2. Acid soluble nucleotides from irradiated and non-irradiated HeLa cells. Dotted lines indicate absorption at 260 mμ. Solid lines indicate activity of P-32.

TABLE I. Relative Specific Activities of Phosphorus Compounds Isolated from Irradiated and Non-Irradiated HeLa Cells.

Peak No.	Compound	Relative spec. activity A. Non-irrad.	Relative spec. activity B. Irrad.	Phosphorus as % of total acid soluble phosphorus (A)	Phosphorus as % of total acid soluble phosphorus (B)
I	?	1.4			
II	Cytidine monophosphate	1.4	1.3	2.1	3.1
III	Diphosphopyridine nucleotide	.4	3.4	1.2	1.6
IV	Adenosine monophosphate	8.0	1.9	.9	1.3
V	?	14.7	18.3	.6	.7
VI	Guanosine monophosphate*	69.7	19.0	.4	1.5
VII	Hexose " *	56.2	53.4	1.5	2.6
VIII	?	30.6	12.4	.3	1.1
IX	?	26.8	8.6	.8	.2
X	Inosine monophosphate*	77.5	92.7†	11.5	22.7
XI	Uridine "	76.4	87.0†	21.0	12.5
XII	Adenosine diphosphate	22.4	26.6	2.2	3.0
XIII	?	4.2	3.1	.6	.7
XIV	?				
XV	?				
XVI	Uridine diphosphoacetylglucosamine*	.65	3.8	4.9	9.8
XVII	" diphosphate glucose*	2.3	2.6	1.4	2.2
XVIII	Guanosine diphosphate*	1.8	3.5	.9	.86
XIX	Cytidine triphosphate*				
XX	Uridine diphosphateglycuronic acid	.6	18.6	.8	.6
XXI	?	5.1	5.1	.6	3.1
XXII	Adenosine triphosphate	22.7	5.7	8.6	1.2
XXIII	Guanosine " *	8.8	6.9		
XXIV	Uridine " *				

* Identity of the compounds has not been proven with certainty.

† These values do not represent true r.s.a. of inosine monophosphate and uridine monophosphate because they were not resolved from inorganic phosphate.

vitro for long periods is found to be quite similar to that of transplanted mouse mammary carcinoma(1) and Ehrlich ascites tumors(2). However, it seems that the proportion of total activity of the acid soluble phosphorus appearing in certain compounds is different. Thus, the unidentified peak XIII seems much more prominent in HeLa cells than in both mammary and Ehrlich cells grown *in vivo*. Relative specific activities of adenosine monophosphate, guanosine triphosphate and uridine triphosphate seem to be lower than in extracts of cells grown *in vivo*. The most prominent effect of irradiation is the reduction of adenosine triphosphate in both amount and activity, indicating a reduction in availability of this form of high energy phosphorus.

That this was likely due to reduction of oxidative phosphorylation is suggested by the increase of activity of the diphosphopyridine nucleotide. The significance of an accumulation of highly radioactive uridine diphosphoacetyle glucosamine in the irradiated cells is difficult to assess.

Summary. Separation of 24 acid soluble phosphorus compounds from irradiated and non-irradiated HeLa cells is described. Relative specific activities of 19 of these compounds are given. Irradiation of the cells produced an increase of relative specific activity and amount of uridine diphosphoacetylglucosamine, and a decrease in amount and specific activity of adenosine triphosphate.

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A Micro-Bioassay Method for Estrogen.* (25994)

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Among responses of the uterus of rat and mouse to estrogen, 2 of the most specific are hypertrophy of the Golgi apparatus and depletion of basal phospholipid in uterine epithelium. These effects have been described (1) in rats which had received estradiol benzoate subcutaneously and earlier observations of lipid depletion have been summarized by Alden(2). The amount of hormone needed to produce these changes has been reduced drastically(3) by administering it by intrauterine injection, a method employed successfully by Hooker and Forbes(4) for progestin assay in mice. Positive estrogenic responses have been produced by this method with as little as 0.00002 μg of estradiol benzoate dissolved in 2 μl of sesame oil when injected into a 10 mm segment of castrate rat uterus.

Materials and methods. A 2 μl volume of test solution was injected by means of a microsyringe into the lumen of a 10 mm segment of one horn of the uterus of 5-months-old Long-Evans rats which had been ovariectomized 14 days. After 48 hr the animals were killed with chloroform, the injected segment removed and divided into 2 pieces and 2 similar tissue samples taken from the contralateral uninjected horn of the uterus. One piece of tissue from each horn was processed by the direct silver method for the Golgi apparatus(5) and the other was fixed overnight in dichromate-sublimate(6), all of the tissue being imbedded in paraffin the following day and cut into 5 μ sections. The Golgi apparatus was available for study immediately after clearing the sections. Phospholipids were visualized by the technic of controlled chromation(6,7,8) for which purpose tissue fixed in dichromate-sublimate and imbedded in paraffin was stained with Sudan black B in 70% alcohol and mounted in Apathy's solution. Glycogen was studied in the dichromate-sublimate fixed tissue by the periodic acid-Schiff technic, with diastase digested controls, followed by nuclear staining with Harris' hematoxylin.

After preliminary experiments to determine the optimum volume of injection fluid and optimum time between injection and autopsy, the effect of graded doses of estrogen was investigated by subjecting castrate rats, in groups of 3, to 2.0, 0.2, 0.02, 0.002, 0.0002 and 0.00002 μg of estradiol benzoate dissolved in 2 μl of sesame oil. For comparison, pairs of rats were given subcutaneous doses of 0.5, 0.4, 0.2 and 0.1 μg of the same hormone with 48 hr intervening before autopsy. Pilot experiments were also conducted with Swiss albino mice.

Results. The changes produced in the Golgi apparatus and basal phospholipid of the uterine epithelium by 0.00002 μg of estradiol benzoate are shown in Fig. 1. With the particular dosage employed, the uninjected horn is essentially unchanged from the castrate condition. The Golgi apparatus of this horn consists of narrow tubules, darkened by silver, occupying a shallow space between nucleus and uterine lumen. In the injected horn the Golgi apparatus has responded by marked hypertrophy. The phospholipid of the uninjected horn is characterized by numerous droplets, stained by Sudan black B, in the basal cytoplasm with subsidiary amounts between nucleus and lumen. In the injected horn the basal phospholipid has been depleted although some droplets remain with this dosage. Between the nucleus and lumen phospholipid of a different significance is present, some of it associated with Golgi apparatus and mitochondria.

Hypertrophy of the Golgi apparatus and depletion of basal phospholipid have proved the most useful criteria of estrogen activity in our experiments since they apply to the mouse as well as the rat and are not subject to distortion by the mechanics of injection or of tissue fixation. In the rat a useful third

* Aided by grant from N.S.F.

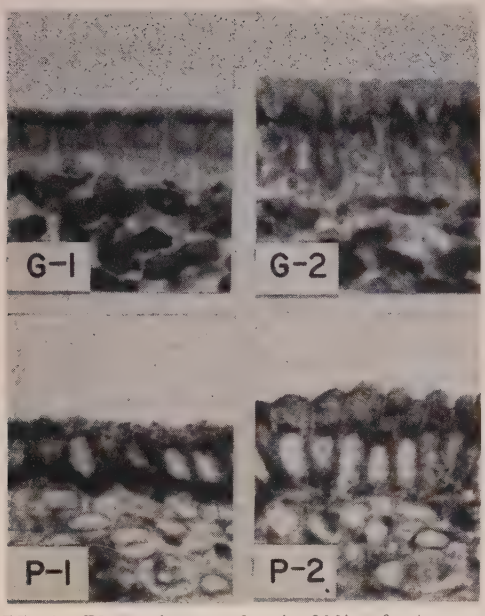

FIG. 1. Photomicrographs (×800) of uterus of castrate rat 48 hr after inj. of 0.00002 μg of estradiol benzoate into 10 mm segment of one horn. G-1 shows Golgi apparatus of epithelium of uninj. horn in the form of narrow black tubules above the nucleus. G-2, inj. segment, has hypertrophied Golgi apparatus. P-1 shows phospholipids of uninj. horn with dense aggregations of black globules basal to the nucleus. P-2, inj. segment, has been depleted of much of the basal phospholipid.

criterion is the appearance of cytochemically demonstrable glycogen(9). Glycogen is found in the longitudinal uterine musculature with a dose of 0.00002 μg of estradiol benzoate. The circular muscle may respond slightly at this dose, especially in the region of the mesometrium, but the epithelium does not respond below a dose of 0.002 μg. The mouse does not give a useful glycogen response but offers instead as a third criterion the mobilization of alkaline phosphatase in uterine epithelium and circular muscle(10). This response also is differential, the epithelium being more sensitive.

Comparison of the uninjected uterine horn with that which received the hormone directly shows that it takes a dose of 0.2 μg before the uninjected horn will present the response evoked in the injected segment by 0.00002 μg. Intrauterine injection is only slightly more effective on the uninjected horn than is subcutaneous administration.

Discussion. Determination of the estrogenic activity of test substances by intrauterine injection allows experiments to be conducted on much smaller amounts of material than has hitherto been possible. This method has additional advantages in by-passing many of the problems of absorption, transportation and distribution inherent in subcutaneous and intraperitoneal administration. It also decreases the confusion which may be introduced by systemic side-effects since the material tested is more nearly confined to the target tissue.

Quantitative measurement of estrogenic potency can be made by testing successively greater dilutions until one is found which produces incipient Golgi hypertrophy, basal lipid depletion and cytochemically demonstrable glycogen in the longitudinal muscle of the rat or alkaline phosphatase in the epithelium of the mouse. The augmentation of these responses which accompanies higher doses may be used for quantitative determinations when methods of measurement have been developed.

Our criteria of estrogenic response have been selected because they involve a minimum of technical skill and produce results which can be evaluated without prolonged training. Many additional cytochemical effects of estrogen can be used and advantage may be taken of increase in epithelial cell height and presence of edema if proper allowance is made for the mechanical interference provided by injection and ligation. Smaller test samples can be handled by employing shorter segments of uterus. This would further increase the sensitivity of the method which is already 10,000 times as sensitive as subcutaneous administration when 10 mm of uterus are used. It is also possible that use of other solvents might enhance the response as it does with intravaginal application(11).

Local application of estrogen to other target tissues has been used in previous assay methods with opening of the vagina in the guinea pig(12) or the appearance of a cornified smear in at least half of the test rats(13) as the criterion of estrogenic activity. The guinea pig test was positive with a minimum dose of 0.0004 μg of estradiol dipropionate

and the cornification test in rats required slightly more than 0.0005 μg of estradiol benzoate, in both cases dissolved in oil. It is possible that the sensitivity of these tests would approach that of intrauterine injection more closely if smaller areas of target tissue could be used or more delicate cytological criteria employed.

The effectiveness of small amounts of hormone when applied directly to the target organ emphasizes their physiological potential. The effect of estrogen is apparent not only in the epithelium but as far peripherally as the longitudinal muscle. Its concentration there must be exceedingly small since our minimum effective dose, if distributed evenly throughout the tissue of the segment into which it was injected, would provide only 4 molecules of steroid for each cubic micron of tissue.

The small amount of test material required has enabled us to carry out successful preliminary experiments with small pieces of tissue excised from organs of estrogenic interest. Although the rat is the more convenient animal for this purpose, it is possible to make simultaneous observations on progestational activity with the criterion of Hooker and Forbes(4) by using the mouse.

Summary. Very small amounts of test substances can be assayed for estrogenic activity by injecting them into the lumen of the castrate uterus of rat or mouse and observing hypertrophy of the Golgi apparatus and depletion of basal phospholipid of the epithelium. Additional criteria are appearance of glycogen in the uterine muscle of the rat and mobilization of alkaline phosphatase in the mouse.

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Uptake of Free Fatty Acids by Skeletal Muscle During Stimulation.* (25995)

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Recent work from several laboratories has suggested that free fatty acids (FFA) of the plasma might be utilized by skeletal muscles (1,2,3,4). However, direct proof of uptake has been hard to obtain, chiefly because of technical difficulties in obtaining blood from the muscles and in measuring blood flow through the muscle. In the present experiments uptake of FFA by the stimulated skeletal muscle was demonstrated in the dog by simultaneous determinations of blood flow and of arterio-venous FFA differences.

Methods. Adult mongrel dogs of both sexes were studied in postabsorptive state under sodium pentobarbital anesthesia (30 mg/kg i.v.). Using the method of Issekutz *et al.* (5), blood flow was determined in the profunda femoris veins which drain predominantly muscular areas. The femoral vein was prepared on the right side and tied 2-3 cm below the junction of profunda femoris veins. A "T" cannula was inserted into the femoral

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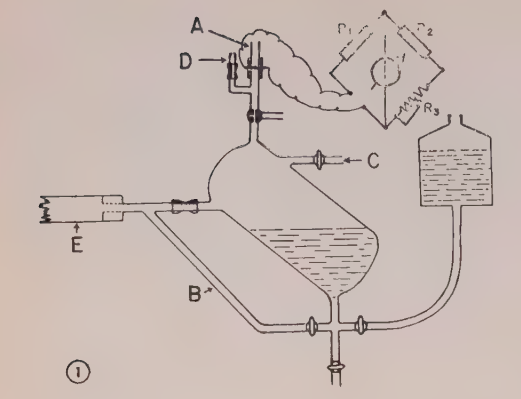

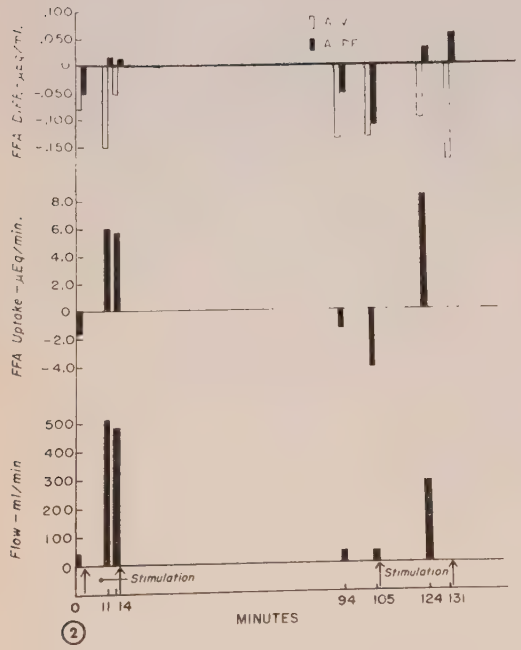

FIG. 1. Schematic representation of apparatus used for blood flow measurements.

FIG. 2. Changes of flow, uptake and A-V difference during repeated stimulation.

vein between the ligature and the deep femoral veins. The long stem of the cannula was connected with a glass tube, 50 cm long and 1 cm wide (Fig. 1, "E"). Cannula and glass tube were filled with physiological saline. The system was kept closed between measurements so that no blood could enter the cannula. Proximally from the profunda femoris veins at about the level of the inguinal ligament, a spring-clamp was applied to the femoral vein, which allowed quick closing or opening of the vessel. All branches of the femoral vein that came from genitalia or abdominal muscles were tied. Closing the clamp forced the blood of profunda femoris veins through cannula into glass tube where it displaced the physiological saline, the level of which was adjusted to provide the necessary pressure gradient. The saline followed by blood entered a glass cylinder at a rate equal to that of the blood flow. The air left the glass cylinder through a hot wire anemometer (Fig. 1, "A") as described by Anrep and Downing(6). A bypass of the air ("D") with empirically selected glass capillaries of various diameters provided a wide range for flow measurements. After reading the deflection of the galvanometer of the Wheatstone-bridge, blood samples were taken from the rubber tubing which connected the cannula with the glass tube. The connection between glass tube and cylinder was then closed and the blood was forced back (*via* "B") by means of compressed air ("C") into the reopened femoral vein, whereby the system was filled again with physiological saline and kept closed until the next measurement. No anticoagulants were used in these studies.

All blood samples were drawn into chilled oxalated tubes, centrifuged, separated and precipitated in the cold. FFA was determined in duplicate analyses using the method of Dole(7).

For direct stimulation of thigh muscles a voltage of 1.3, duration 0.5 millisec. and frequency 10/sec. was used.

Results. Concentration of FFA in the femoral vein was higher than in the femoral artery in agreement with previous findings(8). In 9 out of 11 dogs studied blood from the profunda femoris vein also showed higher FFA concentration than the femoral artery.

Direct electrical stimulation of thigh muscles produced an up to 16-fold increase in blood flow through profunda femoris veins. At the same time, in all but one of the 15 experiments performed on 11 dogs, FFA concentration in profunda femoris veins became lower than in the femoral artery. The veno-arterial FFA difference in the intact, non-stimulated side showed no consistent change during stimulation. No systemic hemody-

namic changes could be detected during stimulation, as judged by blood pressure and heart rate. In the typical experiment shown in Fig. 2, it can be observed that control sample before stimulation shows a negative A-V difference both in intact and cannulated side. During the first stimulation a 16-fold increase in blood flow and a small positive A-V difference developed showing a marked uptake of FFA. Control samples before the second stimulation again showed negative A-V differences on both sides. During the second stimulation blood flow increased about 7-fold with a more marked positive A-V difference on the stimulated side than during first period of stimulation. During both phases of stimulation the negative A-V difference was maintained on the non-stimulated side.

Discussion. In the present studies only an occasional dog showed arterio-venous FFA difference across the skeletal muscle during rest. The lack of more consistency in this respect could be due to the possibility that canine skeletal muscle, unlike human(3), does not remove FFA during rest, or it could be ascribed to the effect of anesthesia. Sodium pentobarbital depresses FFA levels(9) and it also may have a direct effect on metabolism of fatty acids. During direct stimulation of the muscles an uptake of FFA occurred without consistent changes in arterial levels, presumably because the exercise was so well localized, that the other, resting regions of the body compensated for the decrease of FFA in the exercised region.

† Finkelstein, L. J., Spitzer, J. J., Scott, J. C. Unpublished observation.

It is of interest to compare average uptake of 0.1 meq/hr/100 g of skeletal muscle during stimulation with uptake of 0.3 meq/hr/100 g of liver (average of 45 dogs)(10), and that of 0.8 meq/hr/100 g of myocardium (average of 54 dogs).† It should be noted, however, that these values were obtained in different dogs, under slightly different conditions.

Summary. Uptake of free fatty acids by skeletal muscle was determined by simultaneously measuring blood flow through profunda femoris veins, and arteriovenous fatty acid difference. Thigh muscles of the anesthetized dog showed only an occasional uptake of free fatty acids during rest. During direct electrical stimulation of muscles a consistent uptake of fatty acids was found on the stimulated side.

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Utility of 17α-Acetoxyprogesterone in Delaying Estrus in the Bitch. (25996)

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Progesterone has been found capable of inhibiting ovulation in rabbits(1,2), rats(3), guinea pigs(4), mice(5), sheep(6), and cows (7). In the bitch(8), progesterone given by injection will delay estrus for as long as treatment is continued. Progesterone is employed by some veterinary practitioners to delay estrus in dogs but its use is limited as frequent

parenteral treatments are required. In recent years, many synthetic compounds with progestational activity have been prepared and some were found to be active by mouth. In hopes that an orally active synthetic progestin would delay estrus in dogs, 17α-acetoxyprogesterone (Prodox*) was evaluated. 17α-acetoxyprogesterone has been shown to be an orally active progestin and to inhibit ovulation in the rabbit(9). The present work was initiated to determine: (1) if 17α-acetoxyprogesterone would delay estrus in the bitch when administered orally, (2) what dose would be required, and (3) possible deleterious effects of drug administration on the bitch and her subsequent fertility.

Methods. Open Beagle and mongrel bitches weighing approximately 10 kg were employed. For some dogs, 17α-acetoxyprogesterone was incorporated in commercial dog feed checkers (200, 50 and 10 mg/lb feed) and fed *ad lib.* Since each dog consumed about ½ lb of medicated checkers daily, dose of 17α-acetoxyprogesterone was approximately 10, 2.5 and 0.5 mg/kg respectively. For other dogs, 17α-acetoxyprogesterone as a glycerine-sorbitol suspension (25 mg/cc) was administered once daily on the tongue at 4 and 2.5 mg/kg levels. These dogs were fed nonmedicated checkers *ad lib.* Duration of treatments varied from 23 to 70 wks. Estrus was determined by a combination of procedures carried out at weekly intervals. Vaginal smears (stained with 0.1% toluidine blue)(10), gross inspection and palpation of the external genitalia and teasing with stud dogs were employed. Exploratory laparotomies to observe ovaries and uteri were made during the 26th wk of experiment on control dogs and dogs in Groups 2, 4, and 6. Histopathological examinations were made of ovaries and uteri of several dogs that died as a result of evisceration accidents following laparotomies or were killed by penmates during the course of experiment. Whelping data were obtained after completion of treatment schedules. Bitches were bred during either first or second estrual period after cessation of treatment. During the experiment, various chemical and physiological tests were completed on some dogs to characterize possible toxic effects due to administration of 17α-acetoxyprogesterone. These tests were completed during the 6th mo and again during the 16th mo.

* Trade-Mark Reg. U. S. Pat. Off., The Upjohn Co.

TABLE I. Activity of Orally-Administered 17α-Acetoxyprogesterone on Delaying Estrus in Dogs.

Group	No. dogs	Treatment		No. dogs showing estrus
		mg/kg	Duration (wk)	
1	14			14 (100%)
2*	7	10	36	0
3†	3	4	32	0
4*	9	2.5	70	0
5†	3	2.5	32	2 (66.7%)
6*	8	.5	23	7 (87.5%)

* Drug incorporated in feed; fed *ad lib.*
† Drug in suspension; administered once daily.

Results. When 17α-acetoxyprogesterone was incorporated in dog feed and fed at levels of 10 and 2.5 mg/kg, estrus did not occur. Lengths of treatment periods were 36 and 70 continuous wks respectively. At 0.5 mg/kg, estrus was noted in 7 of 8 dogs during a 23-wk treatment period. 17α-acetoxyprogesterone in suspension form administered once daily succesfully inhibited estrus at the 4 mg/kg level in 3 dogs for 32 wks but not at the 2.5 mg/kg level. The 14 control dogs cycled normally at approximately 6-mo intervals (Table I).

Gross *in situ* observations made on ovaries and uteri of control and treated dogs revealed that unregressed corpora lutea were present in ovaries of dogs from Group 1 and 6 but were not present in Group 2 and 4 dogs. Regressed corpora lutea were observed in some dogs in each of these groups. Coiled, corkscrew-like uteri and/or hypertrophied uteri were observed in 80% of the dogs in Group 2, in 44% of Group 4 dogs and in 33% of dogs in Group 6. Uteri of Group 1 dogs were unaltered.

Gross and microscopic examination of ovaries and uteri from treated dogs that died or were killed revealed changes consistent with progestational therapy. Of particular interest were the findings in 3 dogs from Group 2 where average duration of treatment had been 28 wks. Their ovaries were normal grossly. Microscopically, there appeared to be an in-

crease in number of primary follicles. Development of vesicular follicles was inhibited at a rather definite stage (maximum size of 1300 μ) and atresia followed. No evidence that ovulation had occurred was observed. By gross observation, uteri of 2 of 3 dogs were slightly enlarged and displayed several soft, segmented nodules in both horns. Uterus from the third dog was of normal size and shape. Microscopically, the endometrial glandular epithelium in the 2 dogs had undergone hyperplasia with rather extensive papillated mucous-cyst formation. In the third dog, the endometrium was in a relatively quiescent state.

After cessation of treatment, the dogs were maintained to determine time of return to estrus and fertility. Control dogs, Group 1, bred on the third heat after start of experiment, whelped an average of 5 puppies/litter of which 94% were alive at birth. Dogs in Group 2 had first heats an average of 6.5 months after treatment had ceased. These bitches were bred on the first heats, conceived and whelped an average of 5.1 puppies/litter of which 86% were alive at birth. First post-treatment heats in Group 3 dogs occurred at about 1.5 months. The dogs were bred on this first heat and whelped an average of 5.7 puppies/litter with 88% alive at birth. Dogs in Group 4 averaged 2.5 mos to show first heat post-treatment. Four of these bitches were bred on the first heat and 5 on the second heat. In the former group, 2 poor litters, 1 pseudopregnancy, and 1 death occurred whereas in the latter group, 4 puppies/litter were whelped of which 83% were alive. Whelping data on bitches in Groups 5 and 6 are not applicable since delay of estrus was not accomplished in all cases.

Although the primary purpose of the experiment was to observe hormonal effects, safety studies were conducted simultaneously in some of the dogs. No significant deleterious changes in clinical signs, body weight, blood chemistry, urinalysis values, liver morphology or function, kidney function or necropsy findings occurred in bitches on 10 mg/kg for 6 mos and on 2.5 mg/kg for 16 mos.

Discussion. 17α-acetoxyprogesterone incorporated in dog feed and fed *ad lib.* successfully delayed estrus in bitches at the 2.5 mg/kg or higher levels. The 0.5 mg/kg level was ineffective. In contrast, when 17α-acetoxyprogesterone was prepared in suspension form and administered once daily, 4.0 mg/kg was required to delay estrus and 2.5 mg/kg was ineffective. Thus the dose of 17α-acetoxyprogesterone required to inhibit estrus in bitches appeared to vary with frequency of administration. Higher dose level was required when treatment interval was lengthened. Explanation for this finding may be that frequent intake of the drug during a 24-hour period as compared with once-a-day intake minimizes peaks and valleys in blood levels. This point merits further study.

Mechanism of action of 17α-acetoxyprogesterone in delaying estrus was not clearly defined in this limited study. However, the microscopic picture observed in ovaries from dogs on long-term treatment suggested that Graafian follicle maturation was inhibited and ovulation was prevented.

Various 17α-acetoxyprogesterone treatments did not affect future fertility or breeding usefulness of the animals. Gross and microscopic changes observed in ovaries and uteri of treated animals apparently were of a temporary nature. The whelping data were good except for a few dogs in Group 4 that were bred on the first post-treatment heat. For maximum fertility, it would appear reasonable to suggest that bitches be bred no sooner than 6 mos after cessation of treatment.

Previously treated bitches returned to estrus with the usual characteristic signs. No aberrant heats were observed. Close synchronization of heats in dogs within each group did not occur. To forecast with accuracy when previously treated bitches will return to estrus, would appear difficult as great variation occurred. Some dogs returned to estrus within 2 wks after treatment ceased and others took as long as 8 mos to return.

Toxicity studies completed during experiment indicated that the drug was completely safe for dogs.

Summary. When tested for ability to de-

lay estrus in female dogs, 17α-acetoxyprogesterone successfully inhibited estrus during 70-wk treatment period. When incorporated in dog feed and fed *ad lib.*, a minimum dose of 2.5 mg/kg was effective. When administered once daily in suspension form, a dose of 4 mg/kg was required. Histopathological examination of ovaries of treated dogs revealed inhibition of development of vesicular follicles. Bitches previously treated with 17α-acetoxyprogesterone returned to cycle, were bred and whelped normal, healthy litters of puppies. It was suggested that bitches be bred no sooner than 6 mos after cessation of treatment to insure maximum fertility. Drug was judged completely safe for dogs.

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Calcium Requirements of Various Species of *Azotobacter*.* (25997)

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In a reexamination of the trace metal requirements of *Azotobacter vinelandii* O, Esposito and Wilson(1) demonstrated that under defined conditions a quantitative difference existed in level of calcium ion necessary for its growth on molecular and on ammonium nitrogen. The effect of deficient calcium was primarily on length of the lag phase rather than total growth and was definitely associated with the phosphorus metabolism of the organism(2). When it was later established (3) that this requirement could be met by acetate, ethanol or malate, a survey was undertaken to determine how general these findings were among the Azotobacteriaceae. Over a period of 3 years about 25 strains have been examined with the results summarized in this report.

Materials and methods. Esposito and Wilson(1,2,3) have furnished the essential details of technics employed including the special methods for providing a medium with known concentrations of the calcium ion. Further information is given in the thesis of J. A. Bush(4). In most trials urea was used as a source of combined nitrogen to eliminate the complication of change in pH which accompanies utilization of ammonium salts by many of the strains. These are listed in Table I. The inoculum was grown on the modified Burk's medium(1) with no or 20% of Ca^{++} normally added. Size of inoculum was usually 0.1% so maximum calcium carryover was 1.2×10^{-7} M in comparison with the 6×10^{-4} M $CaSO_4 \cdot 2\ H_2O$ (23.4 ppm Ca^{++}) used in the complete Burk's medium.

Results. Calcium requirements of different strains. To present the results obtained with all the strains listed in Table I is neither practical nor necessary; typical findings observed with a single strain of 3 of the species are

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TABLE I. Strains of *Azotobacter* spp. Tested.*

Species	Strain and source
A. vinelandii	O (Wis.); KS-4 (J. O. Harris); 7484, 7496 (ATTC); C-1 (J. P. Voets); B (D. Burk); 3-A (D. B. Johnstone)
agilis	S-1, S-3, S-4 (Wis.); 144 (D. B. Johnstone)
chroococcum	A-3, A-5, VTI (J. P. Voets); 44 (R. L. Starkey)
beijerinckii	B-1, B-2, B-3, B-5 (J. P. Voets)
macrocytogenes	(H. L. Jensen)
indicus	(R. L. Starkey)

* Because of disagreement over taxonomy of Azotobacteriaceae(5,6) nomenclature used by the research workers who kindly furnished us with the indicated cultures has been retained.

illustrated in Fig. 1-3. These data together with those of the other strains may be summarized:

Azotobacter vinelandii (Fig. 1). Although all cultures tested showed an absolute Ca^{++} requirement for growth with atmospheric nitrogen, limited growth was noted in some strains after a lag of 100 to 200 hr. Growth occurring after the lag period was not consistent either between strains or with the same strain. Omission of the calcium ion in the combined nitrogen medium resulted in a slightly extended lag period and a reduction in total growth. The extent of these effects also varied between strains.

Azotobacter chroococcum. Of all species tested, *A. chroococcum* consistently had the most marked requirement for calcium ion when fixing gaseous nitrogen. Cultures without added calcium did not grow even after 2 weeks incubation, and if growth then occurred the cells differed morphologically from those of the inoculum. The old cells were heavily vacuolated, swollen, and variable in their gram reaction. In the combined nitrogen medium without added calcium, results were similar to those obtained from the *A. vinelandii* strains, namely, a slightly extended lag phase and reduced total growth.

Azotobacter beijerinckii. The organisms in this group were the only ones which required addition of calcium ion for growth in both forms of nitrogen media. Cultures with no added calcium did not begin to grow for at least 60 hr while some strains tested required 80-120 hr to initiate growth. Addition of 2.4×10^{-6} M calcium to the combined nitrogen medium shortened the lag period and allowed limited growth, while 2.4×10^{-5} M calcium resulted in a normal growth curve. In the nitrogen free medium 1.2×10^{-5} M added calcium was stimulatory and 2.4×10^{-4} M was necessary for normal growth.

Azotobacter macrocytogenes. Omission of calcium from both forms of nitrogen resulted in an extension of the lag phases similar to results obtained with *A. beijerinckii.* However, *A. macrocytogenes* would begin growth in media without calcium before *A. beijerinckii.* The growth on combined nitrogen without calcium ion was characterized by heavy polysaccharide production.

Azotobacter agilis (Fig. 2). Strains of this species exhibited a consistent growth pattern, in that addition of calcium to either form of nitrogen media allowed only better total growth while omission of the ion did not affect length of lag period or rate of growth.

Azotobacter indicus (Fig. 3). Lag phases of up to 80 hr on both nitrogen-free and combined nitrogen media, were observed with the 0.1% inoculum used in this study. The calcium ion was found to be inhibitory to growth of this organism on both forms of nitrogen. Final pH of the cultures was between 5.0 and 5.5 as compared with 6.8-7.2 for the other species.

Replacement of calcium requirement. Three strains of *A. vinelandii* (O, 7484, 7496) were tested for their ability to use acetate and ethanol in place of calcium when fixing N_2. Check for possible calcium contamination in the acetate was made by adding concentrated HCl to an aliquot of the stock solution of sodium acetate, boiling to remove acetic acid, neutralizing with NaOH and making up to original volume with deionized water. The residue solution was added to control flasks at the same level as were the acetate solutions. Acetate does replace, at least in part, the calcium requirement of these strains (Fig. 4, Table II). No growth occurred in the medium to which acetate residue was added, or in medium with no added calcium, indicating that the medium and acetate stock solutions

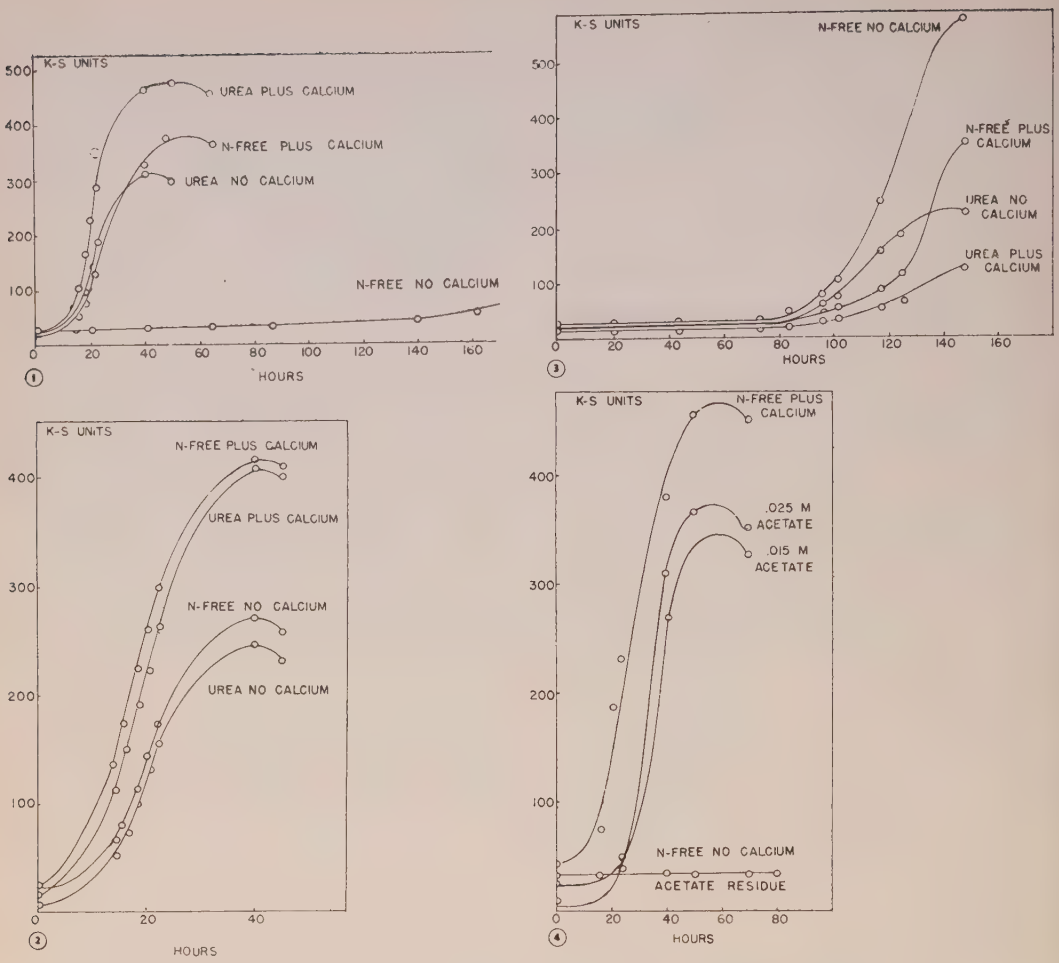

FIG. 1. Effect of calcium on growth of *Azotobacter vinelandii* KS4 in nitrogen-free and nitrogen containing media. Legends in Fig. 1-3 are: N-free + calcium = Burk's medium. N-free, no calcium = Burk's medium minus added calcium. Urea + calcium = Burk's medium + .015 M urea. Urea, no calcium = Burk's medium minus added calcium + .015 M urea.

FIG. 2. Effect of calcium on growth of *Azotobacter agilis* S-3 in nitrogen-free and nitrogen containing media.

FIG. 3. Effect of calcium on growth of *Azotobacter indicus* in nitrogen-free and nitrogen containing media.

FIG. 4. Effect of acetate as a calcium replacing factor in *Azotobacter vinelandii* 7484. N-free + calcium = Burk's medium. N-free, no calcium = Burks' medium minus calcium. .025 M acetate = Burk's medium minus calcium + 1.0 ml 2.5 M acetate/100 ml. .015 M acetate = Burk's medium minus added calcium + 1.0 ml 1.5 M acetate/100 ml. Acetate residue = Burk's medium minus added calcium + 1.0 ml of 2.5 M acetate residue solution.

contained insignificant amounts of calcium ion. Since final pH of the cultures to which acetate had been added was between 8.5-9.0 and since some strains of *A. vinelandii*, not here reported, showed replacement with lower acetate final concentrations (0.0075 and 0.0015 M), but only limited replacement with higher acetate final concentrations (0.025 and 0.015 M), it is possible that the pH rise caused by liberation of sodium ions was inhibitory to growth at the higher acetate concentrations. Ethanol also replaced the calcium ion for nitrogen fixation, although less total growth and lower fixation levels were obtained.

Discussion. The results in Fig. 1-3 suggest that species of the azotobacter may be associated into 3 groups according to their re-

TABLE II. Effect of Acetate and Ethanol as Calcium Sparing Factors on Nitrogen Fixation by *Azotobacter vinelandii*.

Media	Final nitrogen, μg/ml	
	Strain O	Strain 7496
Burk's medium	280	265
Burk's medium minus added calcium	25	20
Idem plus .025 M acetate	275	230
Burk's medium minus calcium plus 1.3×10^{-4} M ethanol	135	

quirements for Ca^{++}. Group I consists of those strains in which a definite quantitative difference is required for growth on free and combined nitrogen. It is represented in this study by strains of *A. vinelandii* and *A. chroococcum* for which the difference is marked and by those of *A. beijerinckii* (and possibly *A. macrocytogenes*) in which the difference observed was only one order of magnitude. Group II represented by strains of *A. agilis* are those in which no requirement for added calcium was demonstrable when grown on either free or fixed nitrogen, *i.e.*, the purified medium contained sufficient Ca^{++} for their needs on either source. Group III consists of the single strain of *A. indicus* tested for which the calcium ion is definitely inhibitory independent of source of nitrogen. Whether these differences are sufficiently consistent among the various species to be useful for taxonomic purposes cannot be determined in view of the relatively small number of strains used. Some investigators(5), however, have urged creation of a new genus (*Beijerinckii*) for *A. indicus* because of its physiological differences, including inhibition by Ca^{++}, from the other species of the Azotobacteriaceae.

While these studies were in progress Norris and Jensen(7,8) published results of a similar survey that included several of the strains used in this investigation. Although there is a large area of agreement among the findings from the two laboratories, two discrepancies deserve comment. First, Norris and Jensen were unable to confirm our results of replacement of calcium by acetate(3) when a small inoculum was used. In our original study we used 2% of a 24 hr culture containing about 50 μg N/ml so that initial concentration was approximately 1 μg N/ml. We have verified this finding using 0.1% inoculum (0.05 μg N/ml) using 3 strains of *A. vinelandii* (Fig. 4, Table II) and have also confirmed it with strain O using only 0.01% inoculum.

Probably of greater significance was the inability of these workers to detect a quantitative difference in requirement for Ca^{++} on N_2 and NH^{+}_4 –N for strains of *A. vinelandii* including our strain O. This strain has been repeatedly and independently tested by a number of workers in our laboratory over a period of several years with essentially the same results—about the only difference we have noted has been the level of calcium necessary to obtain normal growth with respect to lag, turbidity, or total nitrogen. This will vary as the experimental conditions change, but always a range can be found in which a marked difference in response on free and combined nitrogen is observed. For example, when size of the inoculum is varied from 0.01 to 2%, length of the lag on both sources of nitrogen markedly increases, but a range of Ca^{++} concentration is readily established in which growth on combined nitrogen is essentially normal whereas during this same period of time, little if any growth occurs on N_2. The obvious criticism that, in spite of all efforts to purify the medium, contaminating calcium plays a role, misses the point that a medium can be prepared with its level of calcium so controlled and other conditions (size of inoculum, temperature and time of incubation) so defined that marked differences in growth response of this strain on free and combined nitrogen can be repeatedly and consistently obtained.

Summary. Calcium ion requirements of strains representing 5 species of the Azotobacteriace have been studied. Quantitative differences in growth response on molecular nitrogen and on urea-N were readily detected with strains of *A. vinelandii* and *A. chroococcum* and less definitely with *A. beijerinckii* and possibly *A. macrocytogenes*. No specific requirement for calcium was detected with strains of *A. agilis*, and the ion inhibited growth of *A. indicus* on either source of nitrogen. Acetate and ethanol replaced the cal-

cium required for nitrogen fixation by 3 strains of *A. vinelandii* tested.

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Effect of Epinephrine on Experimental Ectopic Ventricular Tachycardias.* (25998)

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One is occasionally faced with the problem of a patient requiring administration of catecholamines while at the same time exhibiting ectopic cardiac rhythms. The most striking example is seen in shock accompanying acute myocardial infarction and not infrequently associated with ventricular extrasystoles or tachycardia. The investigations on action of epinephrine during chloroform anesthesia(1) as well as later reports on epinephrine-induced ventricular fibrillation while heart was under the influence of benzol, cyclopropane or halogenated hydrocarbons have warned the clinician not to use catecholamines in the course of certain ventricular arrhythmias, the danger being appearance of ventricular fibrillation. At symposium on action of catecholamines on the heart, held in Burlington, Vt., we mentioned the effects of intravenous injections of epinephrine in dogs during ventricular tachycardias(2). This is the report on these experiments.

Method. Twenty-nine dogs were anesthetized with intraperitoneal injections of Nembutal (18 mg/kg) and morphine (8 mg/kg). Morphine was given to increase vagal tonus thus preventing a sinus tachycardia interfering with ectopic rhythms. For the same reasons the vagi were left intact. Artificial respiration was instituted and the heart was exposed. The electrocardiogram was registered in lead 2. Ventricular arrhythmias were provoked by sub-epicardial injection of either 0.05 cc of a 20% solution of sodium chloride, a 3.8% solution of sodium oxalate or application of a few crystals of delphinine to the surface of one of the ventricles. Epinephrine hydrochloride was injected into the superior caval vein in the amount of 0.01 cc/kg as soon as a ventricular tachycardia with a constant rate appeared.

Results. The data from these experiments are shown in Table I. In none of these experiments did ventricular fibrillation occur after intravenous injection of epinephrine nor did epinephrine produce any of the dangerous prefibrillatory arrhythmias. In the tachycardias provoked by sodium chloride in the right ventricle the rate increased after epinephrine in 13 of 25 experiments; it remained unchanged 6 times and decreased in 6 experiments. Data from the left ventricle are similar. In 14 experiments the rate increased 10 times, it was unchanged in 2 experiments and became slower twice after intravenous injection of epinephrine. When sodium oxalate was used to create ventricular tachycardia, the rate increased after epinephrine injection 4 times, decreased once and remained unchanged 3 times. In 5 experiments epinephrine was injected during a ventricular tachycardia elicited by topical application of

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TABLE I. Ventricular Rates before and after Injection of Epinephrine.

Date	NaCl on right ventricle		NaCl on left ventricle		Delphinine on right ventricle		Sod. oxalate on right ventricle	
	Before	After	Before	After	Before	After	Before	After
9/29/59	272	300	250	275				
10/ 6			214	214				
13	230	250	220	250	187	272		
	240	250						
20					193	187		
27	214	230	206	214				
11/10	230	230	222	228			156	171
							214	214
17	214	214					187	214
24	250	250						
12/ 1	187	230						
8	187	200	250	250				
15	214	187					206	187
22	138	251						
1/ 5/60	272	276						
12	214	214					200	200
19	230	214						
26	206	214						
2/ 9	200	187	230	206				
16	200	150	214	275				
30	200	225	206	275				
3/22			175	214			138	230
29			206	250				
4/ 5	214	200						
12	187	230	230	222				
19	150	214	214	240			136	200
5/ 3	160	230						
10	230	250			200	136		
17	240	214	230	250	206	275		
24	214	214					214	214
6/14	230	230	300	300	250	300		

delphinine. The rate increased in 3 experiments and diminished in 2.

The increase of rate amounted often to a few beats per minute and only exceptionally was it remarkable (Table I). In only 4 experiments did new extrasystoles appear at the peak of epinephrine action each time originating in the ventricle opposite the one from which the tachycardia originated. In all experiments varying types of A-V heart block appeared at end of the tachycardia due to reflex vagal effects secondary to the rise of blood pressure. Tachycardias never lasted more than 2 minutes and 20 seconds and were not definitely prolonged by epinephrine. When ventricular tachycardia was suppressed by a sinus tachycardia it was possible to reestablish it temporarily by faradic vagus stimulation and inhibition of the atria.

The disappearance of experimental ectopic rhythms under the influence of epinephrine or faradic stimulation of cardiac sympathetic nerves has been reported with aconitine extrasystoles(3), in ventricular arrhythmias provoked by acetyl strophantidine(4) and it was found that in 9 out of 16 experiments epinephrine protected the heart from fibrillation provoked by electrical stimulation in presence of acetylcholine(5). A longer duration of atrial action potential because of a slower repolarisation rate under epinephrine(6) may be responsible. The dual effect of epinephrine in the present experiments, the increase and decrease of rate of tachycardia is certainly related to the complex action of the drug; vulnerability of the heart was briefly enhanced, then depressed when epinephrine or norepinephrine was given(7).

These experiments do not demonstrate the safety of use of catecholamines in presence of ventricular ectopic rhythms. While epinephrine abolishes an existing ventricular tachy-

cardia it may provoke other ectopic rhythms. It may, however, be concluded, that when catecholamines are clinically indicated, the presence of a ventricular ectopic rhythm should not be a deterrent.

Summary. Intravenous injection in the dog of epinephrine in the amount of 0.01 cc/kg in presence of a ventricular ectopic tachycardia, did not elicit ventricular fibrillation in 54 attempts. The tachycardia was moderately increased in most experiments or slowed down. Only in 4 experiments did the rate rise for more than 50 beats/minute.

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Glucuronic Conjugation of Steroids in the Avian Adrenal Gland.* (25999)

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It is well established that adrenal steroids are conjugated with glucuronic acid as one phase of their inactivation prior to excretion in mammals. Furthermore, the liver is generally regarded as being the primary site of this conjugation, enzymatic control of which has been ascribed to 2 enzymatic systems: UDPG dehydrogenase (in particle-free fraction)(1, 2) and a glucuronosyl transferase (in microsomes)(3,4,5).

During another study, evidence was obtained indicating that a considerable portion of total steroid content of the avian adrenal tissue was in a bound form—conjugated with both glucuronide and sulfate(6). This report presents data which support the previous finding by demonstrating that the avian adrenal gland possesses the enzymatic systems responsible for both conjugation of adrenal steroids with glucuronide and hydrolysis of glucuronide-bound steroids.

Methods. The adrenal glands used to measure glucuronide formation were obtained by dissection from 3-4 month old White Leghorn cockerels immediately after decapitation, weighed and homogenized in ice-cold Krebs-Ringer-PO_4 buffer (pH 7.4) with a Potter-Elvehjem homogenizer.

Replicate fractions of the homogenate were incubated in Warburg flasks at 37°C for 2.5 hours with one of the following steroids† as a substrate: 11-dehydro-17-hydroxycorticosterone (E, cortisone), 17-hydroxycorticosterone (F, cortisol), pregnane-3α,17α,21-triol-11,20-dione (H_4E, tetrahydrocortisone), or pregnane-3α,11β,17α,21-tetrol-20-one (H_4F, tetrahydrocortisol). Each flask contained: 0.5 ml uridine diphospho-glucuronic acid (UDPGA) (100 γ/ml); 2.0 ml homogenate (equivalent to 80-100 mg wet tissue); 1.0 ml steroid (100 γ/ml $\cong$ .27 μmoles; and 4.5 ml Krebs-Ringer-PO_4 buffer containing 90 mg glucose/ml, making a total volume of 8 ml.

Prior to incubation of the homogenate with substrate, 4 ml was removed from each flask and boiled to inactivate enzymes. These 4 ml portions were left at room temperature to function as unincubated controls.

After incubation, the flasks were placed in boiling water to inactivate enzymes. Both incubated and nonincubated control samples were then extracted 3 times with methylene

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† Cortisone, tetrahydrocortisone and tetrahydrocortisol were generously supplied by Merck Inst. through the courtesy of Dr. H. J. Robinson.

chloride and steroid concentration of the pooled extract determined by the Porter-Silber technic(7) with the use of a Beckman DU spectrophotometer. For each set of determinations from one pooled homogenate, a control flask containing 8 ml buffer was treated similarly to the incubated samples and used to set the spectrophotometer prior to reading the other samples.

Subtracting the amount of free steroid in the nonincubated samples from that of its corresponding incubated sample yielded the amount of free steroid which disappeared during incubation.

The contents of both incubated and nonincubated flasks were next adjusted to pH 5.0 with 0.2 M acetate buffer, 1 ml of β-glucuronidase (*Ketodase*) was added and the samples hydrolyzed at 47°C for 18 hours. Samples were then reextracted with methylene chloride and free steroid determined as before. The difference in free steroid content between the originally incubated and nonincubated flasks gave the amount of steroid liberated in hydrolysis with β-glucuronidase.

To prepare a microsomal fraction, adrenals were homogenized in an icecold suspending medium containing 300 g sucrose, 2 g Versene (disodium ethylenediamine tetraacetate) and 1.0 g Tris (trishydroxy methyl amino methane) buffer in 1000 ml distilled H_2O. The suspension was centrifuged at 10,000 g for 15 minutes in a Servall refrigerated (1-2°C) centrifuge. Nuclei and mitochondria were removed and discarded. After centrifugation at 105,000 g for 1 hour in a Spinco Model L ultra centrifuge, the supernatant was discarded and the microsomes resuspended in the above medium so that 1 ml of the resulting suspension was equivalent to 10 mg protein/ml.

To 1 ml of the microsomal preparation were added 0.7 ml of one of the 4 steroids (100 γ/ml) ≅ .19 μmoles, 0.5 ml UDPGA (100 γ/ml) and 0.8 ml Krebs-Ringer-PO_4 buffer. Replicates of this mixture were incubated at 38°C for 30 minutes(4). Corresponding control mixtures without UDPGA were run concurrently.

Following incubation the tubes were boiled to inactivate the enzymes and extracted with chloroform to remove unbound steroid and the steroid determined by the Porter-Silber method. After adjusting pH to 5.0 the residual material was incubated with β-glucuronidase (*Ketodase*) for 18 hours at 47°C and reextracted with chloroform to remove liberated steroid which was determined by the Porter-Silber technic.

Prior to extraction after incubation, 0.3 ml of original incubation mixture was removed and UDP content determined by measuring the disappearance of reduced DPN in the phosphopyruvate-pyruvate kinase system coupled to the DPNH-lactic dehydrogenase system(8).

β-glucuronidase activity was determined in the adrenals from young White Leghorn cockerels by a slight modification of the method of Talalay *et al.* using phenolphthalein glucuronide as a substrate(9). An aliquot of homogenized tissue was mixed with substrate and buffer at pH 5.5. After incubation for 1 hour at 37°C the tubes containing the mixture were placed in boiling water for 5 minutes to inactivate the enzyme and precipitate protein prior to development of the color. A unit of β-glucuronidase was arbitrarily designated as that quantity of enzyme responsible for hydrolysis of 1 γ substrate per hour under the above conditions.

Results. The results of incubating avian adrenal homogenates with compounds E, F, H_4E or H_4F in presence of excess UDPGA are shown in Table I. In each case except E, a statistically significant amount of the steroid substrate disappeared during incubation as compared with an equal number of unincubated control determinations ($P < .001$ for H_4E and H_4F; $P < .025$ for F). The percent decrease in steroid content for the 3 compounds varied from 13.7% for F to 27% for H_4F. The 2% decrease for E probably can be attributed to experimental error since the statistical probability was = .53.

Following hydrolysis with β-glucuronidase and reextraction, free steroid, representing the steroid previously bound with glucuronide, was recovered (Table I). A discrepancy exists in the case of compound E where twice as

TABLE I. Steroid Conjugation.

Preparation	Substrate	No. determinations	Steroid* disappearing on incubation	Steroid* recovered as glucuronide	UDP* recovered
Total homogenate	E	10	.008	.017	
	H_4E	14	.053	.011	
	F	10	.037	.009	
	H_4F	10	.073	.016	
Microsomal fraction	E	7	0	0	0
	H_4E	17	.040	.020	.021
	F	6	.023	.008	.014
	H_4F	11	.013	.010	.011

* μmoles in total incubated mixture.

much glucuronide-bound steroid was recovered as disappeared on incubation with UDPGA; an adequate explanation of this result is not readily apparent. For the other 3 compounds, the amount of hydrolyzed steroid varied from 20.7% (H_4E) to 24.3% (F) of the amount that disappeared in the original incubation. Of the 4 compounds used, statistically significant results ($P < .025$) were obtained only in the case of F.

Since total homogenates of adrenal tissue undoubtedly contain a vast assortment of endogenous enzymes and steroids other than the added substrate, it is not surprising that the results of incubating microsomal fractions from avian adrenal glands with steroid substrates offered more clear-cut results (Table I). The percentage of the added steroid substrate which disappeared during incubation with microsomes was the greatest for H_4E (21.0%) and least for H_4F (6.8%) and E (0%). After hydrolysis with β-glucuronidase, 34 to 77% of the 3 steroids which had disappeared during the initial incubation was recovered as glucuronide-bound material. Amount of UDP formed during conjugation was measured and found to correspond reasonably well with the amount of recovered glucuronide-bound steroid.

Since in the procedure using microsomes the UDP formed so closely matched the quantity of glucuronide formed, it is probable that the discrepancy between the steroid that disappeared upon initial incubation and that recovered subsequent to hydrolysis is due to a fairly large share of the steroid substrate having been metabolized by enzymatic mechanisms leading to products other than glucuronide formation and/or products other than those reactive to the Porter-Silber technic. This possibility also exists with use of total adrenal homogenates.

It is generally held that glucuronide conjugation of Δ^4-3-keto-steroids is preceded by consecutive irreversible saturation of the 4-5 double bond under the influence of Δ^4-3-keto-steroid hydrogenase(10,11,12) and irreversible reduction of the C_3 carbonyl group by 3-hydroxysteroid dehydrogenase (13). If this generalization holds for the microsomal system used in the present study, the explanation for the fact that F but not E appeared to be conjugated must mean that F was converted to H_4F prior to conjugation. Alternatively, it would have to be assumed that conjugation of F occurred at a position other than C_3(4,14). Unfortunately, our data do not permit a decision on either alternative.

The results of determining β-glucuronidase in avian adrenal tissue (and in liver and kidney for comparison) (Table II), indicate that adrenal tissue contains almost as much of this enzyme as does kidney and 23% as much as does liver. Furthermore this concentration of β-glucuronidase in the adrenal was stable under various chronic treatments (hydrocorti-

TABLE II. β-Glucuronidase Content of Adrenal, Hepatic and Renal Tissue.

Age (days)	No. birds	β-glucuronidase* Adrenal	Liver	Kidney
24	9	1.38 ± .1	5.34 ± 1.1	1.51 ± .1
14	7	1.42 ± .1	5.52 ± 1.1	2.28 ± .4
16	8	1.23 ± .2	6.29 ± 1.5	1.71 ± .3
14	8	1.53 ± .2	6.27 ± 1.6	2.14 ± .1
14	10	1.39 ± .1	5.84 ± .9	1.71 ± .3

* Units/mg tissue. A unit is that amount of enzyme responsible for liberating 1 γ phenolphthalein at 38°C, at pH 5.5, in 1 hr.

sone, cortisone, amphenone, hyypophysectomy, reserpine, hexestrol, O′, P′-DDD, restraint) some of which altered the concentration in liver(15).

Although the hepatic enzyme systems responsible for glucuronide formation have been studied extensively in mammals and identified in the pigeon(16), no data are available concerning their presence in adrenal tissue. β-glucuronidase, responsible for hydrolysis of glucuronides, has been reported to have a wide distribution in various organs (including the adrenal) in both mammals and birds. The presence of the enzymes controlling both synthesis and hydrolysis of steroid glucuronides in adrenal tissue, together with the demonstration that a large percentage of glucuronide-bound steroid occurs in the avian adrenal during normal and experimental conditions (6,15), strongly suggests that the steroid glucuronides play a role in adrenocortical metabolism in the bird. The nature of this role remains to be investigated.

Summary. Total homogenates and microsomal fractions of avian adrenal glands were incubated with cortisone, cortisol, hydrocortisone or hydrocortisol in the presence of excess uridine diphospho-glucuronic acid. The resulting data indicate the presence of an enzyme system in the microsomes capable of conjugating all the above compounds except cortisone with glucuronide with liberation of uridine diphosphate. The presence of β-glucuronidase was also demonstrated in avian adrenal tissue. These results, together with the previous demonstration that significant amounts of glucuronide-bound steroids are present in the avian adrenal gland under normal and various experimental conditions, imply that steroid glucuronide conjugation and hydrolysis play some role in the normal physiology of this gland.

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Effect of Warfarin (Coumadin) Sodium Administration During Lactation on Blood Coagulation of Nursling Rats.* (26000)

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The anticoagulant warfarin (Coumadin) sodium has come into wide use for treatment and prevention of various thromboembolic disorders, including those in postpartum patients(1,2). This latter application has raised the question of the drug's effect in lactation, *i.e.*, whether or not anticoagulant therapy for the mother would have an adverse effect on the nursling. This problem has now been investigated by determination of whole blood clotting time by the capillary method in mother rats fed diets containing warfarin

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TABLE I. Toxic and Therapeutic Warfarin Sodium Dosage Levels.

Warfarin sodium, % diet	Mothers		Nurslings	
	Survival	Clotting time, min.	No.	Clotting time, min.
.01	1/3	>390	3	>260
.005	3/5	>100	13	38 (10 –140)
.001	2/5	>100	22	6.4 (4 – 10)
.0005	2/2	50 (10 –90)	9	3.1 (1 – 10)
.00025	3/3	5.7 (4.5– 7)	33	1.1 (1 – 1.5)
.000125	11/11	3.1 (3 – 3.5)	87	1.0 (.5– 1.5)
None	11/11	1.1 (.5– 1.5)	75	1.0 (.5– 1.5)

sodium, and in their nurslings.

Materials and methods. The rats used were CF albinos (Carworth Farms). In the various experiments mothers were alternately put on stock diet or warfarin sodium (WS) diet on the same day that the litters were born. The stock diet was ground Purina Lab Chow checkers. WS diets were prepared by dissolving the anticoagulant in acetone, adding the solution to a small amount of ground stock diet, grinding well in a mortar, and finally mixing the concentrate thoroughly with the required amount of stock diet in a Hobart mixer. Food consumption was recorded. The experimental diets were fed for 7 or 14 days. The experiments could not be carried beyond 14 days because after that age the nursling rats were large enough to crawl near the solid diet. At the end of experimental periods mothers and nurslings were etherized, and blood samples were taken by exposing the heart, snipping the left ventricle with a scissors, and filling a capillary glass tube† (0.04 mm I.D., 90 mm long) with the freely flowing cardiac blood. Whole blood clotting time was determined by breaking the capillary tubing at intervals until a fibrin thread was observed. Intervals of 15 seconds were used in the principal experiments, and longer intervals in the exploratory work. All determinations were made at the controlled room temperature of 25 ± 1°C. Prothrombin determinations were made on some of the mother rats, blood sample being drawn from the heart by syringe. The Quick one-stage method(3) was used, but with a nichrome stirring loop to get the plasma clotting endpoint.

† Progressive Laboratory Specialities Co., Jamaica, N. Y.

Results. Determination of toxic and therapeutic dosages. In Table I are shown results of the 7-day range-finding experiments. At a WS level of 0.01% of the diet, only 1 out of 3 mothers survived, and the survivor had a tremendously prolonged clotting time of more than 390 minutes. Survival incidence in their nurslings was not meaningful, because of the poor condition and deaths of the mothers. However, the 3 surviving nurslings all showed greatly prolonged clotting times, greater than 260 minutes, and gross evidence of hemorrhage beneath the skin of the neck and at other sites. This demonstrated that at WS levels highly toxic or lethal for the mother, the anticoagulant action was transmitted through the milk. Gross hemorrhage was also seen in 2 of the 13 surviving nurslings at the next lower level of 0.005%, but was not seen below that dosage.

The anticoagulant effect in both mother and nurslings decreased with decreasing dosage. However, at all dosage levels there was much less anticoagulant effect on nurslings than on mothers. Thus, at 0.00025% WS all 33 nurslings had normal clotting times of 1.0-1.5 minutes, whereas the 3 mothers had an average clotting time of 5.7 minutes (range 4.5-7), or about 5 times normal. At the dosage level of 0.000125% clotting time of the mothers was 3.1 minutes (range 3-3.5), which was 3 times the average normal and double the 1.5 minute upper limit of normal clotting range. According to Connell and Mayer(4), the therapeutic range for clinical anticoagulation is about 1.5-2 times normal whole blood clotting time by their method. Therefore, the WS level of 0.000125% was considered to be at least equal to therapeutic anticoagulation of the mothers and was selected for further investigation and analysis.

TABLE II. Comparison of Clotting Times of Mothers and Nurslings at Therapeutic Warfarin Sodium Dosage Level.

Warfarin sodium, % diet	No. days	Mothers		Nurslings	
		No. animals	Clotting time, min.	No. animals	Clotting time, min.
None	7	11	1.1 ± .1 *	75	1.02 ± .07*
.000125	7	11	3.1 ± .1	87	.97 ± .04
None	14	16	.95 ± .07	123	.97 ± .04
.000125	14	16	3.1 ± .1	120	.95 ± .04

* Mean ± S.E.

Comparisons at therapeutic dosage. At the therapeutic WS dosage level of 0.000125% clotting times of mothers and their nurslings were compared after 7 days and after 14 days of feeding. In Table II are given mean clotting times and standard errors. In both experiments clotting time of the WS mothers was about 3 times that of the stock diet control mothers. By the "t" test these differences were found to be highly significant statistically ($P = <0.001$ in each experiment). However, clotting times of the WS nurslings were normal, in fact, very nearly the same as those of control nurslings. Clotting time for the WS mothers was practically the same after 14 days as after 7 days, indicating that the 0.000125% dosage was essentially a stabilized maintenance level. Prothrombin activity was determined on a group of 10 WS mothers and averaged about 25% (range 15-32%) of the levels in mothers on normal stock diet, thus corresponding to a therapeutic or prophylactic level. At the dietary WS level of 0.000125%, average daily food consumption of the nursing mothers was about 25 g, equivalent to a WS intake of approximately 0.16 mg/kg of body weight. No significant pathology was found in WS mothers or nurslings, when autopsied after clotting time had been taken.

Growth and reproduction of warfarin sodium nurslings. Further studies were made on the nurslings to determine whether or not there would be delayed deleterious effects. After 14 days on the therapeutic level of 0.000125% WS, 4 mothers were transferred to stock diet and allowed to complete weaning of their litters. Likewise, 4 control mothers on the stock diet were allowed to wean their litters. The male and female WS nurslings showed normal weaning weights, growth, and reproduction (Table III). The second generation young in turn were weaned and grew normally. On autopsy at termination of experiment, the parents and the second generation young appeared normal. Thus, ingestion of the therapeutic anticoagulant level of WS by lactating mothers produced no delayed deleterious effects in offspring.

Discussion. A certain amount of confusion has existed regarding the effects on the young of anticoagulant administration to the mother during pregnancy and during lactation. In clinical and animal studies with the older anticoagulant, bishydroxycoumarin, it was indicated that the fetus or newborn was more susceptible to anticoagulant action than the mother(5). Special caution is recommended in using anticoagulants antepartum(6). When bishydroxycoumarin was administered to lactating rats, bleeding was caused in the nursing young; however, the dosage used was so high that it was lethal to the mothers(7). It was concluded from later experiments in dogs that it would probably be quite safe to administer bishydroxycoumarin to the mother during lactation because little of the anticoagulant gets

TABLE III. Growth and Reproduction of Nurslings from Mother Rats Fed Therapeutic Level of Warfarin Sodium.

Warfarin sodium in diet, %	At weaning (3 wk)					Reproduction			
	No. litters	No. young	Avg wt of young, g	Avg wt gain in 9 wk, g ♂	Avg wt gain in 9 wk, g ♀	No. mothers	No. litters	No. young	Avg weaning wt, g
None	4	46	31	238	153	12	12	84	37
.000125	4	37	34	274	161	11	11	97	37

into the milk(5). In subsequent clinical studies of nursing mothers, it was reported that therapeutic dosages of bishydroxycoumarin could be administered without danger of hemorrhage or effect on prothrombin time in the nursing infants(8,9). The present experiments on warfarin sodium in rats are consistent with the above results, and suggest that therapeutic dosages of warfarin sodium could probably be administered to nursing mothers without deleterious effect on the infants.

Summary. When a therapeutic dosage of the anticoagulant warfarin (Coumadin) sodium was fed to lactating rats, there was no effect on whole blood clotting time of the nurslings, or on their subsequent growth and reproduction. When toxic or lethal levels of warfarin sodium were fed to the mothers, clotting time was prolonged in the nurslings. This demonstrated that the anticoagulant effect could be transmitted through the milk when mothers received toxic dosages. However, at all dosages the anticoagulant effect was much less in nurslings than in mothers.

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Site of Formation of Thyroglobulin in Mouse Thyroid as Shown by Radioautography with Leucine-H^3.* (26001)

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Locating sites of protein synthesis in the thyroid gland has been attempted after injection of S^{35}-labelled methionine(1,2), but may now be done better with tritium (H^3)-labelled amino-acids, since the soft beta-rays of tritium allow a more precise radioautographic localization than those of S^{35}. When mice were injected with methionine-, glycine- or leucine-H^3, the location of the H^3 label in the thyroid was found to be the same with the 3 amino-acids but reactions were more intense with leucine-H^3 than with the other 2. This last result was not unexpected since leucine has been listed(3) as the most abundant amino-acid in thyroglobulin, the characteristic iodinated protein found in the thyroid colloid. The next step was to investigate quantitatively the thyroid gland of those mice which had been treated with leucine-H^3. This was done in the present investigation.

Methods. Ten adult male mice weighing 26-32 g received a single injection of 5 μc of DL-leucine-4, 5-H^3 (150 mc/mM) per gram body weight and were sacrificed in pairs at ½, 4 and 35 hours, 7 and 45 days later. Thyroid glands were fixed in Bouin, stained with hematoxylin-eosin and radioautographed(4).

In one section of each thyroid gland, the largest and smallest diameters of the colloid of every follicle were measured with a micrometer; and photographic grains were counted within a 10.4 x 10.4 μ area over the center of the colloid. Grains were then counted within 2 x 10.4 μ areas selected over 2 diametrically opposed portions of the epithelium of each follicle. Finally, grains were also counted in 10.4 x 10.4 μ areas located outside the thyroid

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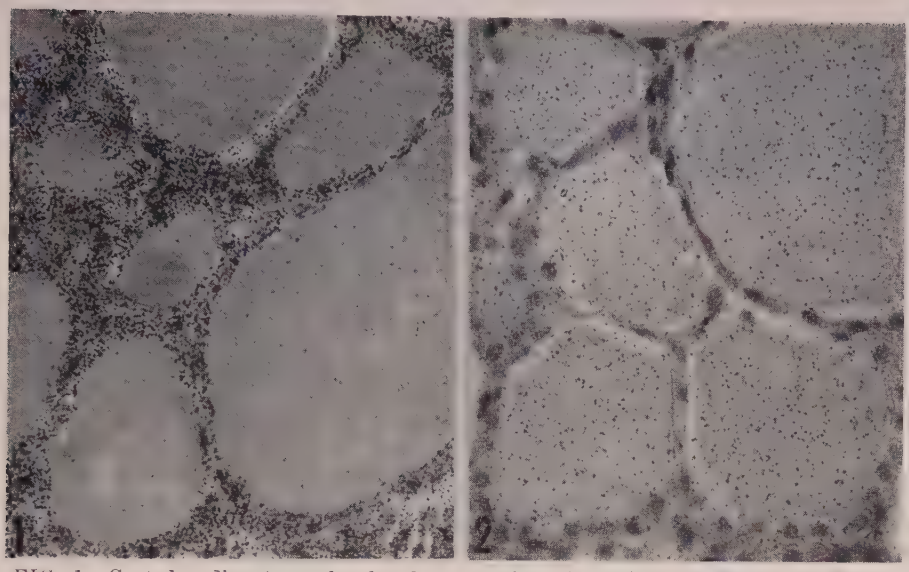

FIG. 1. Coated radioautograph of a hematoxylin-eosin stained section of thyroid gland from an adult mouse, sacrificed ½ hr after inj. of leucine-H^3. Abundant silver grains overlie epithelium but not colloid of thyroid follicles. (The rare grains found over the colloid are attributable to the background fog of the emulsion.)

FIG. 2. As in Fig. 1, except the animal was sacrificed 35 hr after inj. of leucine-H^3. Grains over colloid are much more abundant than at early time interval (Fig. 1).

gland, as an estimate of the background fog of the emulsion to be deducted from the counts over colloid and epithelium.

The grain counts expressed per unit area were grouped for follicle sections in which mean colloid diameter was 20 μ or less, 21-40, 41-60, 61-80, 81-100, 101-120 or greater than 120 μ. Since the diameters of the follicle sections did not necessarily represent the true diameters of the follicles from which they were derived, the grain counts were corrected as done previously(5)‡ and referred to 6 follicle classes (Tables I and II).

TABLE I. True Concentration of Leucine Label in *Epithelial Cells* of Thyroid Follicles, from Radioautographic Grain Counts.

Colloid diam., μ	Grain counts per unit area at various times after admin. of radio-leucine				
	½ hr	4 hr	35 hr	7 days	45 days
40	69.2	23.8	24.4	—	2.7
60	31.6	21.7	23.1	8.4	4.7
80	36.7	26.9	22.9	7.1	5.0
100	44.2	29.3	24.1	5.8	4.0
120	30.3	—	30.8	10.2	4.4
140	50.4	25.0	20.1	10.0	4.4

TABLE II. True Concentration of Leucine Label in *Colloid* of Thyroid Follicles, from Radioautographic Grain Counts.

Colloid diam., μ	Grain counts per unit area at various times after admin. of radio-leucine				
	½ hr	4 hr	35 hr	7 days	45 days
40	.7	12.1	28.0	—	1.6
60	.6	4.8	15.3	9.8	7.9
80	1.1	5.2	10.3	5.4	6.9
100	1.4	4.0	3.1	5.3	5.3
120	—	5.2	4.8	15.1	5.8
140	1.5	2.7	5.2	4.3	3.7

Results. At a half hour after injection, the leucine label was present throughout the epithelium of every thyroid follicle (Fig. 1), while at 35 hours and later it appeared in the colloid (Fig. 2).

The corrected or "true" concentration of label in the cells at any given time interval (Table I) was not significantly influenced by diameter of the follicles. Concentration was maximal at a half hour after injection and decreased with time, rapidly between ½ and 4 hours, and slowly thereafter.

The true concentration of the label in the colloid (Table II) was insignificant at the half hour interval, then increased with time in all follicle classes. A maximum was reached at 35 hours in the 3 smaller follicle classes.

‡ Raw data and methods used in calculations reported in this article will be supplied on request.

By 45 days, radioactivity had decreased in all follicles.

Discussion. Following administration of radioactive amino-acids, the radioactive label appears in newly-formed proteins(6), site of synthesis of which can be demonstrated by radioautography(1). Accordingly, the radioautographic reaction observed over the epithelium of all follicles soon after a single injection of leucine-H^3 (Fig. 1) demonstrated that all epithelial cells of the thyroid gland were synthesizing protein.

The radioautographic grain count over the epithelium decreased after ½ hour, indicating loss of the newly-formed protein (Table I). Analysis revealed that this loss occurred rapidly in an early phase (turnover time, 7.5 hours) and more slowly thereafter (turnover time, 19.7 days), suggesting production in the epithelium of at least 2 types of new protein.

During the early phase, the decrease in concentration of labelled protein in the epithelium coincided with an increase in the colloid (Table II). Calculation of radioactivity in the entire epithelium and colloid at ½ and 35 hours revealed that epithelial content decreased by 135 and colloid content increased by 117 arbitrary units of radioactivity between the 2 time intervals. The rather close correlation implied that the loss of labelled protein from the epithelium could be accounted for by simultaneous appearance of labelled protein in the colloid. The small relative difference between the figures for epithelium and colloid, if at all significant, may mean that not all the labelled substance turned over by the epithelium in the first phase was deposited into the colloid, and/or some of the labelled substance deposited in the colloid had already been itself turned over in this interval.

By virtue of the similarity between the properties of isolated thyroid colloid(7) and purified thyroglobulin(8), it is believed that the main constituent of thyroid colloid is thyroglobulin. And, since leucine has been reported to be the most abundant amino-acid in thyroglobulin(3), the leucine-containing protein synthesized by the cells, then rapidly released to the colloid, must be the precursor of thyroglobulin. Now, the organic binding of iodine in the thyroid takes place in the colloid(9,10); hence, the formation of typical thyroglobulin (containing the iodinated amino-acids from which thyroid hormone is derived) must occur after the precursor reaches the colloid.

The much less rapid turnover of the label which took place in the epithelium during the later phase (turnover time of 19.7 days) had no similar counterpart in the colloid. Rather, the slow loss was similar to that reported for cells of many organs and was likewise attributed to the internal turnover of cellular proteins(1). It was estimated that about one-half of the labelled protein made in the epithelial cells was thus used up in intracellular metabolism, while the other half consisted of the thyroglobulin precursor to be deposited into the colloid.

Analysis of the turnover of leucine label in the colloid of each follicle size class suggested presence of only one type of labelled substance, that is, newly-formed thyroglobulin (whatever its degree of iodination). While cells of all follicle classes seemed to synthesize precursor at about the same rate (Table I), a greater rate of concentration of newly-formed thyroglobulin was observed in the colloid of small follicles as opposed to large follicles (Table II), due to the fact that there is a greater volume of epithelium per unit volume of colloid in smaller than in larger follicles (5).

The eventual decrease with time in colloid content of labelled protein in all follicles (Table II) may be attributed to the proteolytic breakdown of thyroglobulin (as a result of which thyroid hormone is liberated(11).

Summary. Ten adult male mice were injected with tritium-labelled leucine and sacrificed in pairs at various time intervals thereafter. Thyroid gland sections were radioautographed by the coating technic and photographic grains counted over epithelium and colloid. The leucine label is incorporated into protein by every cell of every thyroid follicle at 30 minutes after injection, but its concentration in the cells decreases thereafter. Concentration of labelled protein in the colloid was insignificant at ½ hour, increased and reached a maximum at 35 hours or soon

thereafter, and subsequently diminished. The appearance of the leucine label in colloid of all follicles examined between ½ hour and 35 hours could be accounted for by simultaneous loss of label from the epithelial cells. Since leucine is the most prominent amino-acid in thyroglobulin(3), the results are interpreted to represent the synthesis of this protein in the epithelium and its secretion into the colloid.

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6-Beta-Hydroxy-Cortisol: High Levels in Human Urine in Pregnancy and Toxemia.* (26002)

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6-beta-hydroxy-cortisol (6β-OH-F) has been shown by Burstein to exist as a major metabolite of cortisol in guinea pig urine(1). He also detected its presence in minute amounts in normal human urine and in larger amounts in urine excreted during late pregnancy and after cortisol administration to normal males(2,3). Colle and Ulstrom have recently found significant quantities of 6β-OH-F in urine of the human newborn(4). The high degree of polarity conferred by the extra hydroxyl group renders 6β-OH-F difficult to extract from aqueous media with many conventional organic solvents and greatly hinders its chromatographic separation in systems designed for less polar corticosteroids. To overcome these difficulties, new methods of extraction and chromatography have been devised and the following experiments were performed using such technics.

Methods and materials. Fresh unhydrolyzed urine is extracted with ethyl acetate after prior addition to the urine of 20% by weight of sodium sulfate; this salt is also added in similar concentrations to the alkali and aqueous washes which follow. Recovery experiments and determination of partition coefficients validated the efficiency of extraction of 6β-OH-F by this method. Chromatography is carried out in 2 successive highly polar solvent systems, the first using chloroform:ethyl acetate:methanol:water; and the second, benzene:*tert.*-butanol:water. After elution from the second paper, quantitation is accomplished by the Porter-Silber reaction. Hydrolysis of the urine with β-glucuronidase before extraction did not increase the yield in several experiments. Identity and homogeneity of the 6β-OH-F recovered were confirmed by a number of technics in which an authentic sample was compared; these include chromatographic mobilities and color reac-

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‡ Fellow of National Foundation, 1959-60.

§ Deceased.

TABLE I. Urinary Steroids in Third Trimester of Pregnancy.

	Total P-S chromogens, μg/24 hr	No. of patients	Allo-THF, THE, μg/24 hr	No. of patients
Non-toxemic	3810 (3200-4600)*	4	2850 (1860-3230)*	4
Toxemic	4080 (1730-6500)	4	2590 (400-5160)	6
Non-pregnant	4150		3470	

* Mean and range.

tions of the isolated substance and its chemical derivatives, sulfuric acid absorption spectra, and infrared spectroscopy in some cases.

Determinations of urinary 6β-OH-F were made in 3 groups of subjects: normal men and women between the ages of 23 and 45, normal women in the third trimester of pregnancy, and pregnant women in the third trimester with pre-clampsia or toxemia of pregnancy. In addition to the 6β-OH-F determination, total corticoids were measured by the Porter-Silber reaction on another aliquot of urine following β-glucuronidase hydrolysis and ethyl acetate extraction. These latter extracts in some cases were chromatographed on a Bush B_5 system, and combined tetrahydrocortisol (THF)§, allotetrahydrocortisol (allo-THF),‖ and tetrahydrocortisone (THE)¶, were measured by methods previously described(5).

Results. 6β-OH-F values are shown graphically in Fig. 1. Mean values and ranges in μg/24 hours were: normal males, 436 (309-510); normal females, 373 (181-760); normal pregnancy, 845 (563-1320); toxemic pregnancy, 1970 (1490-2390).

Values for total Porter-Silber corticoid excretion and combined metabolites after chromatography are listed in Table I together with mean values for non-pregnant subjects obtained in this laboratory.

Other steroidal substances having similar polarities but occurring in lesser concentrations were also noted on the chromatograms. One of these was tentatively identified as an isomer of 6β-OH-F, 6α-hydroxycortisol.

Discussion. Several interesting findings emerged from these experiments. First, levels of 6β-OH-F in normal adults were considerably greater than those of any other unconjugated hormone we observed, including cortisol itself, which, as reported by others, averages about 40 μg per day(6,7).

Second, a definite rise in 6β-OH-F excretion occurs in the third trimester of normal pregnancy which is not related to any general rise in corticoid excretion, resulting in an increased ratio of 6β-OH-F to the other metabolites measured. This suggests that there is an increased conversion of endogenous cortisol to 6β-OH-F in normal pregnancy rather than an increased total production of cortisol.

Third, the increased concentrations of 6β-OH-F seen in normal pregnancy become further intensified in toxemia. A slight rise in overall Porter-Silber steroid excretion in the toxemic group, too small here to be significant, might be accounted for, if further confirmed, by increased ACTH production under conditions of stress. Both this factor and altered steroid degradation may be operating to increase the 6β-OH-F excretion in toxemia.

An alternative possibility is that 6β-OH-F arises not as a degradation product of cortisol but as an independent secretion of the adrenal gland, since incubation studies by Touchstone (8) indicate that the human adrenal gland is capable of producing this hormone *in vitro*.

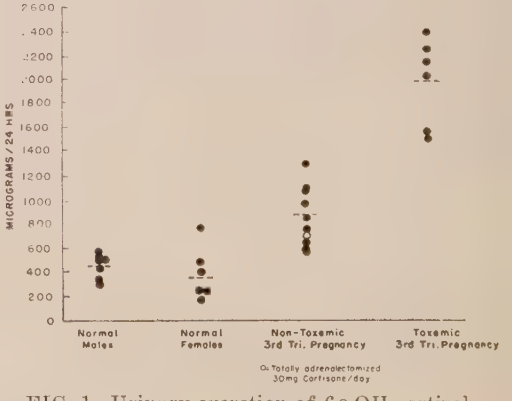

FIG. 1. Urinary excretion of 6β-OH-cortisol.

§ THF, 3α,11β,17α,21-tetrahydroxy-pregnan-20-one.

‖ allo-THF, 3α,11β,17α,21-tetrahydroxy-allopregnan-20-one.

¶ THE, 3α,17α,21-trihydroxy-pregnane-11,20-dione.

Some preliminary experiments have been carried out in feeding large doses of exogenous cortisol to subjects in each of the 3 groups studied and measuring % of dose recovered as 6β-OH-F. There was 13.5 and 16% conversion to 6β-OH-F in 2 toxemics and 13.5 and 22% in 2 non-toxemic pregnant women, while 2 non-pregnant women excreted 3.8 and 6% as 6β-OH-F. These observations indicate that there is an increased conversion of cortisol to the 6-beta derivative in both toxemia and normal pregnancy.

The biological effects of 6β-OH-F have not been fully studied, but at present there is no evidence which would assign to it a causative role in toxemia. The likelihood is that 6β-hydroxylation represents an available pathway for cortisol degradation, rendering the steroid more water soluble and suitable for excretion without the necessity for A-ring reduction and glucuronide conjugation. If the enzymatic steps concerned with either of these 2 reactions are interfered with, as might occur in some conditions, then 6-beta-hydroxylation could function as an important alternate pathway of cortisol metabolism.

Summary. 6β-OH-cortisol has been found to be the most abundant unconjugated corticoid in human urine. Normal pregnancy is accompanied by elevated levels which are further increased in toxemia. The evidence to date suggests that in these conditions an altered metabolism of cortisol takes place in which 6β-hydroxylation becomes of greater quantitative significance.

We acknowledge with thanks Dr. Seymour Bernstein's generous gift of the 6β-OH-F standard. Infrared spectroscopy was performed through the kindness of Dr. Samuel Solomon and Dr. Seymour Lieberman.

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Immunological Differentiation of Human Testicular (Spermatocele) and Seminal Spermatozoa.* (26003)

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The seminal plasma and spermatozoa of man have powerfully antigenic material in common(1). These antigens are highly organ and species specific. The same is true for rabbit(2), guinea pig(3), bull(4), and buffalo(5). The fact that similar antigens are found in aqueous extracts of prostate and seminal vesicles, but not in those of testicle or epididymis, and that the ejaculate of azoospermic man contains these antigens, suggested that they are the product of the adnexal glands of the genital tract and that spermatozoa take up the antigens during their passage through these organs(1,2). Recently, direct evidence was obtained that spermatozoa from the rabbit's epididymis lack these antigens(6).

For men, similar evidence is difficult to obtain, because spermatozoa from testes or epididymis cannot be easily obtained in adequate numbers. An alternative source for spermatozoa, however, is occasionally available in

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spermatoceles. These cyst-like structures form when the epididymis becomes obstructed (7,8). Usually they contain very numerous spermatozoa, indistinguishable in morphology from seminal spermatozoa and often found motile at the time when a spermatocele is punctured or opened by surgical intervention. Inasmuch as these spermatozoa are excluded from contact with the adnexal glands, they can for our purposes be considered as equivalent to testicular spermatozoa.

The fluid content of spermatoceles is low in protein. Immunologically, these proteins are indistinguishable from those of the serum and do not cross-react with seminal plasma or spermatozoa(9).

We were fortunate in obtaining spermatocele contents through courtesy of Dr. John R. Herman. Thus we were able to procure direct evidence that in man also testicular spermatozoa lack the antigens present in seminal spermatozoa and in seminal plasma.

Materials and methods. Three specimens of spermatocele content were available for these experiments:

a) From a 56 year old man; 218 ml of spermatocele fluid was collected containing 17 x 10^9 spermatozoa (77 x 10^6/ml).

b) From a 45 year old man; 93 ml spermatocele fluid containing 5 x 10^9 spermatozoa (54 x 10^6/ml).

c) From a 40 year old man, 2 ml containing 19 x 10^6 spermatozoa.

A minority of the spermatozoa was found motile on examination immediately after collection.

The spermatozoa were removed from the spermatocele fluids by high speed centrifugation, washed thrice in physiological saline solution, and resuspended in the same diluent in a concentration of 10^8 cells/ml. Dilutions were made from this in saline solution as needed. Sterile precautions were observed. Merthiolate was added to a concetration of 1/10,000.

Immune sera against human seminal spermatozoa, seminal plasma, whole serum, or aqueous organ extract were available from previous work. New batches of immune sera were prepared as needed according to the methods previously described(1,2,5). Rabbits and guinea pigs were injected with spermatozoa from spermatoceles in Freund's adjuvant according to these procedures. In these publications, the methods of complement fixation test (Kolmer modification) and precipitation by the agar diffusion method of Ouchterlony used for the work presented here have also been given.

Results. Spermatozoa from spermatoceles did not react with immune sera against seminal spermatozoa or seminal plasma (Table I). They also did not react with anti-human serum immune sera.

Seminal spermatozoa remove antibody both from anti-seminal plasma and anti-seminal spermatozoal sera. Absorption of antibody can be demonstrated in the agar diffusion test, if a suspension of spermatozoa in proper concentration(or seminal plasma) is added to a trough containing immune serum. Spermatocele spermatozoa do not inhibit formation

TABLE I. Fixation of Complement by Rabbit Immune Serum (Diluted 1/10) and Antigens (Human).

	A		B		C		D		E	
	Spermatocele spermatozoa 2×10^6/0.1 ml		Seminal spermatozoa 2×10^6/0.1 ml		Seminal plasma 1/200		Spermatocele fluid 1/100		Blood serum 1/100	
Antigen dilution	Anti-sem.	Anti-sperm.	Anti-sem.	Anti-sperm.	Anti-sem.	Anti-sperm.	Anti-sem.	Anti-sperm.	Anti-sem.	Anti-sperm.
1/1	2+	2+	4+	4+	4+	4+	—	—	—	—
1/2	—	—	4+	4+	4+	4+	—	—	—	—
1/4	—	—	4+	4+	4+	4+	—	—	—	—
1/8	—	—	—	—	4+	4+	—	—	—	—
1/16	—	—	—	—	—	4+	—	—	—	—
1/32	—	—	—	—	—	—	—	—	—	—

Antiserum and antigen controls, also antigens plus normal rabbit serum negative.
Anti-sem. = Anti-seminal plasma serum; Anti-sperm. = Anti-seminal spermatozoal serum.
4+, no hemolysis; 2+, moderate (approx. 50%) hemolysis; —, complete hemolysis.

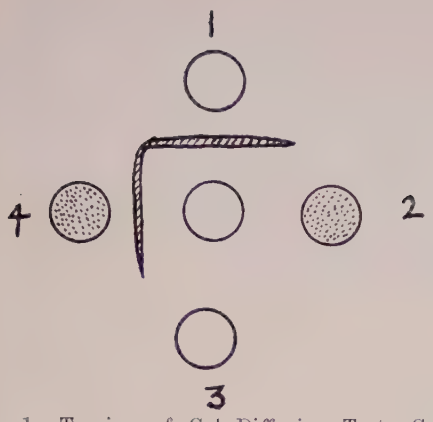

FIG. 1. Tracing of Gel Diffusion Test. Center trough: Human seminal plasma. Peripheral troughs all contain anti-seminal plasma immune serum; (1) no addition; (2) 10^8 seminal spermatozoa (in 0.05 ml saline) added; (3) 0.05 ml seminal plasma added; (4) 10^8 spermatocele spermatozoa (in 0.05 ml saline) added.

of a line of precipitation between the trough containing immune serum and the one with antigen (seminal plasma) (Fig. 1); seminal spermatozoa or seminal plasma inhibit precipitation. Thus the first agent lacks the antigenic material present in the two others.

We have not been able thus far to obtain evidence of antibody formation in sera of rabbits or guinea pigs treated with spermatozoa from spermatoceles. However, immunization experiments with this antigen are being continued in rabbits, guinea pigs and mice.

Discussion. Immunological evidence is presented showing that spermatozoa from spermatoceles ("testicular" spermatozoa) lack the antigens which characterize seminal plasma and seminal spermatozoa. Similar data have been previously presented regarding the difference between testicular and seminal spermatozoa of the rabbit(6). Thus the highly antigenic material present on spermatozoa from semen must be taken up during the passage through the adnexal glands. Though we have failed hitherto to obtain evidence of antigenicity of spermatozoa obtained before they come into contact with the secretions of the adnexal glands (collected from spermatoceles), the data of Henle(10,11) of Voisin (12) and Freund(13) show that testicular spermatozoa are not devoid of antigenicity, and those of Pernot(3,4) show that such antigenicity may still be discerned in seminal spermatozoa. At the present time, it remains unknown whether the antigenic material acquired by spermatozoa during their passage through the adnexal glands plays any role in the physiology of reproduction.

Summary. Spermatozoa from spermatoceles lack the antigenic material present on seminal spermatozoa, which these latter cells share with the seminal plasma. This provides direct evidence that human spermatozoa acquire antigenic material during their passage through the adnexal glands of the genital tract.

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ECHO and Poliomyelitis Virus Antisera Prepared in Guinea Pigs with Fluorocarbon-Treated Cell Culture Antigens. (26004)

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The preparation of virus antigens and antisera by cell culture grown virus has led to difficulties in using these sera in the complement fixation (CF) test, since the fixation between cell culture host antigens and antibodies may interfere with the specific fixation between virus antigen and antibody. The authors(1) and other investigators(2,3,4) have shown that host antigen CF activity can be removed from many viral CF antigens prepared in cell cultures by fluorocarbon treatment, without loss of specific virus CF antigenicity. Although a simple procedure, fluorocarbon treatment would be more convenient for many purposes if it could be used for preparation of the immunization antigens, in which case untreated CF antigens could be used with these antisera.

This report will present data on virus and host CF antibody titers in guinea pigs immunized by fluorocarbon-treated cell culture antigens of ECHO and poliomyelitis viruses.

Material and methods. Virus strains. The following virus strains were used to prepare immunization and CF antigens: prototype strains of ECHO types 1 through 20(5,6), "Mahoney," "MEF-1" and "Saukett" strains of poliomyelitis types 1, 2, and 3, respectively. *Cell cultures.* Monkey kidney cell cultures obtained from Microbiological Associates, Bethesda, Md., were grown in a medium containing 2% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Earle's solution. At time of inoculation, the cultures in 32 oz prescription bottles were washed twice with Hanks' solution and 40 ml of maintenance medium containing 2% guinea pig serum (for immunization antigens) or 2% calf serum (for CF antigens), 0.5% lactalbumin hydrolysate, and 97.5% Earle's solution was added.

Immunization antigens. Monkey kidney cell cultures were inoculated with 0.3 ml undiluted virus. The bottles were incubated at 37°C. After complete degeneration of cells had taken place the fluids were harvested and treated once or twice with fluorocarbon† by the method reported previously(1). Each fluorocarbon-treated antigen was titrated in the CF test against 4-8 antibody units of anti-monkey kidney guinea pig serum to test host antigen activity. The immunization antigen of normal monkey kidney cells was prepared by mechanically removing cells from monkey kidney cultures. The cells, 3 times centrifuged and washed, were used as a 50% solution for immunization.

The antigens were stored at –20°C. All antigens containing penicillin were treated with penicillinase before being used in immunization of guinea pigs.

Immunization of animals. Groups of 4 guinea pigs were immunized 3 times each with 1.0 ml of each antigen and bled 1 week after last injection. The first and second injections were given subcutaneously, at an interval of 3 weeks. The last injection, 4 weeks after the second, was given intracardially or intraperitoneally.

Complement-fixing antigens. CF antigens were prepared as immunization antigens except that they were not treated with fluorocarbon. The specific CF activities of the antigens of ECHO types 1 through 14 and 3 poliomyelitis types were tested with homologous antisera prepared in monkeys(7) and ECHO type 20 with control positive human serum. A "normal" monkey kidney cell antigen was prepared by inoculating a monkey kidney cell culture with Coxsackie B type 5 virus.

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† Genetron 113, obtained from Allied Chemical & Dye Corp., New York.

TABLE I. Host and Virus CF Reactions of Fluorocarbon-Treated and 2 Untreated Immunization Antigens and Corresponding CF Antibody Titers in Pooled Sera of 2 to 4 Guinea Pigs. Only groups, which, in each of the 3 injections, received an antigen with no host CF reaction, are listed. 4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

Immunization antigen	Fluorocarbon treatment	Reaction of immunization antigen with: Anti-monkey kidney G.P. serum, Antigen dilutions			Homologous monkey antiserum, Antigen dilutions			Reciprocal of CF antibody titer in pooled G.P. antisera	
		1:1	1:2	1:4	1:1	1:2	1:4	Host	Virus
ECHO- 1	+	0	0	0	4	4	1	32	512
2	+	1	0	0	4	4	2	<16	128
6	+	0	0	0	4	2	0	32	1024
6	—	4	4	3		ND		512	2048
8	+	0	0	0	4	2	0	<16	128
13	+	0	0	0	4	1	0	64	2048
14	+	0	0	0	4	1	0	<16	512
14	—	4	4	4		ND		128	512
15	+	0	0	0		ND		<8	512
16	+	0	0	0		ND		<8	256
17	+	0	0	0		ND		<8	512
Polio- 1	+	0	0	0	4	ND	ND	<8	1024
2	+	0	0	0	4	ND	ND	<8	1024
3	+	0	0	0	4	ND	ND	<8	512

ND = not done.

CF test. Complement fixation tests were performed by the standard technic used in this laboratory(1).

Results. Twenty-one groups of guinea pigs were immunized by fluorocarbon-treated ECHO and poliomyelitis virus, but only 12 groups (ECHO types 1, 2, 6, 8, 13, 14, 15, 16, 17, and 3 poliomyelitis types) received, in each of the 3 injections, an antigen with no host antigen activity in the CF test. The others were immunized at least once with an antigen from which host CF antigen activity had not been removed. The results of the former group (no host antigen activity) are shown in Table I, where host and virus CF antigen activity of the immunization antigens and corresponding host and virus CF antibody titers in pooled antisera of 2 to 4 guinea pigs are recorded. Nine of the 12 antigens produced no host CF antibodies in guinea pigs at dilutions tested (1:8 or 1:16). However, 3 immunization antigens (ECHO types 1, 6, 13) with no host activity in the CF test produced host antibodies in titers of 1:32 or 1:64. Host antibody titers of guinea pigs immunized with non-treated antigens were 1:128 or 1:256. All 12 fluorocarbon-treated antigens produced specific virus antibodies in titers of 1:128 or more. The difference between virus and host antibody titers was 16-fold or more in antisera prepared with fluorocarbon-treated antigens and 2-fold with non-treated antigens.

The virus and host CF antibody titers of all ECHO and poliomyelitis guinea pig antisera are shown in Table II. As was indicated above, in preparation of many of the ECHO antisera, at least one injection consisted of an antigen with some host CF antigenicity left. Titers of all sera against "normal" monkey kidney antigen were 1:64 or less. Titers against homologous virus CF antigen were between 1:16 and 1:2048. The difference between titers against homologous and "normal" monkey kidney antigen was 16-fold or greater in 15 out of the 20 ECHO types, and in all poliomyelitis types, 8-fold in 2 ECHO types (9 and 10), and 4-fold or less in 3 ECHO types (3, 7, and 19).

No heterotypic CF reactions were found in poliomyelitis antisera. Heterotypic reactions within ECHO types 1, 8, and 13 were the same as reported previously(1,6,7).

Discussion. The results presented indicate that fluorocarbon-treated cell culture grown ECHO and poliomyelitis virus could be used as immunization antigen to prepare antisera in guinea pigs with no host CF antibodies. Although some fluorocarbon-treated antigens with no host CF antigen reaction produced

TABLE II. Reciprocals of Homologous and Host CF Titers, and Some Heterologous Titers of ECHO Type 1-20 and Polio Type 1-3 Guinea Pig Antisera Prepared by Immunizing Animals with Fluorocarbon-Treated Antigens.

	Guinea pig sera																							
CF antigen	ECHO- E-1	E-2	E-3	E-4	E-5	E-6	E-7	E-8	E-9	E-10	E-11	E-12	E-13	E-14	E-15	E-16	E-17	E-18	E-19	E-20	Polio P-1	Polio P-2	Polio P-3	Anti-monkey kidney
ECHO- 1	512							0			0	0	512											
2		128																						
3			128																					
4				512																				
5					256																			
6						1024																		
7							64																	
8	512							128					256											
9									512															
10										512														
11	0										512													
12												256												
13	512							0				0	2048											
14														512										
15															512									
16																256								
17																	512							
18																		128						
19																			16					
20																				1024				
Polio-1																					1024	0*	0*	
2																					0*	1024	0*	
3																					0*	0*	512	
"Normal" monkey kidney	16	0	32	0	0	32	32	0	64	64	32	0	64	0	0*	0*	0*	8	16	16	0*	0*	0*	256

0 — <16 0* = <8

host antibodies on repeated immunization of guinea pigs, these were present in such low titers that on simple dilution the antisera would show specific activity only. The practical value of some of the antisera prepared for this study has been proved by using them in CF typing of enterovirus isolates(8).

On the other hand, the data presented show that, besides the host activity, the specific activity of fluorocarbon-treated antigens should be tested before using them for immunization. From ECHO types 15-19 no antisera were available during time of immunization. Hence, the lack of specific virus antibodies in ECHO type 19 antiserum may be caused by lack of specific virus activity in fluorocarbon-treated immunization antigen, which could not be tested.

Since each virus group differs in susceptibility to fluorocarbon treatment(4), the fluorocarbon technic can not be adapted directly to preparation of antisera of other viruses, but if adaptable it might be of value.

Summary. It was found that ECHO and poliomyelitis virus antisera without host CF antibodies could be prepared in guinea pigs by immunizing the animals with fluorocarbon-treated cell culture grown virus. Although in repeated immunization of guinea pigs 3 out of 12 fluorocarbon-treated antigens with no host CF activity produced host antibodies in titers of 1:32 and 1:64, specific virus titers were 16 and 32 times higher. It was also found that, besides the host activity, the specific virus activity of fluorocarbon-treated antigens should be tested before using them for immunization.

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Concentrated Culture of Gonococci in Clear Liquid Medium.* (26005)

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The common practise is to grow *Neisseria gonorrhoeae* on solid media, although the organism can be cultivated successfully on liquid semi-defined(1,2) and undefined media (3). These must usually be enriched with blood, starch, charcoal and similar material, however, and the resulting cloudiness complicates the observation of growth. An important growth-limiting characteristic of the gonococcus is its sensitivity to certain amino acids, fatty acids and possibly other toxic materials that are carried with ordinary medium constituents(4), including agar(5). Adsorptive removal of such inhibitory factors apparently accounts for the necessity of adding various enrichment materials. These might be spatially separated from the actual growth environment and still function, provided that diffusional access to the enrichment material is provided. A simple biphasic flask system (6) was so employed and allowed attainment of gonococcal growth in a water-clear men-

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struum and to a population density several times higher than usual.

Methods. The biphasic flask system(6) consisted simply of a thick layer of medium solidified with 2% agar and overlaid with a thin layer of liquid medium. Most of the experiments were done with 100 ml of agar and 25 ml of supernatant broth in a 250-ml Erlenmeyer flask. This method superficially resembles but in principle differs from the double-medium technic of Castaneda(7), which facilitates transfer and detection of colonies on an agar surface.

The medium used was Difco's dextrose-starch formulation consisting of 1.5% proteose peptone No. 3, .2% dextrose, 1% soluble starch, .5% NaCl, .3% $Na_2HPO_4 \cdot 2H_2O$, and 2% gelatin. The final pH was 7.3. The broth was clarified by adding Celite #512 (Johns-Mansville Co.) and filtering while hot through a pad of the same material. Agar was added at a concentration of 1% for direct use and 2% for use as the underlay in the biphasic system.

Seven freshly-isolated strains of culturally proven *Neisseria gonorrhoeae* were obtained from the State Bacteriological Laboratory in Stockholm and were maintained by serial transfer on agar medium. Experimental cultures were inoculated with approximately 1% by volume from a 48- or 72-hour broth culture, but the number of viable cells inoculated was found difficult to standardize. Consequently, the time course of growth could not be compared directly between experiments. Humidified air containing 10% carbon dioxide by volume was maintained over the cultures by connecting them with a reservoir. Since little change in volume of the gas phase was observed, it also was possible simply to stopper tightly the flasks after flushing with the CO_2-air mixture. Incubation was at 37°C on a rotary shaker, which was operated at 100-150 rpm in a radius of 1¾ inches. Cultural purity was checked by Gram staining and streaking on peptone agar.

Growth was measured in nephelometer with a linear decimal scale to 100. Direct counts of formalinized and diluted samples were made by phase microscopy using a conventional counting chamber. Estimates were made of numbers of cells in clumps, and the assumption was made that half of the apparent single cells were doubles. However, degree of multiplicity varied between different methods of cultivation so that at best the method only provided estimates of actual numbers of single cells. The data given represent minimum estimates.

The results of growth in the biphasic system were compared to results in a control flask containing the same total volume of medium. Concentration index expresses how many times more concentrated the organisms are per milliliter in the liquid layer of the biphasic culture than in the control broth culture. Yield index represents the ratio of total number of cells per flask in the biphasic system to total number in the broth control.

Results. A qualitative comparison of growth of 7 strains of *N. gonorrhoeae* first was made in shaken broth flasks, shaken biphasic flasks, static broth tubes, static biphasic tubes, and static agar-slant tubes. Complete dextrose-starch medium with appropriate addition of agar was used in each case. Some growth occurred under all of the conditions, but it was readily apparent that the most rapid, dense and homogeneous liquid growth occurred in the shaken biphasic flasks. Growth of the strains varied from light to very heavy final populations. Typical quantitative results with a representative strain grown in shaken biphasic flasks are given in the first line of data in Table I. Somewhat higher cell density but lower efficiency were obtained using 50 ml of agar overlaid with 12.5 ml of broth; *e.g.* 82 nephelos units, 5.1 x 10^9 single cells per ml, a concentration index of 2.2, and a yield index of 0.44 were obtained after 72 hr incubation.

Since the large molecular weight materials in dextrose-starch broth probably function as adsorptive and neutralizing materials rather than as nutrients, it seemed likely that incorporating them in the agar layer of the biphasic system should provide the same function and that the broth overlay consequently could be simplified. Accordingly, a series of biphasic flasks was made in which components of dextrose-starch broth were omitted from the broth overlay and the layers were allowed to

TABLE I. Growth of *N. gonorrhoeae* Strain 861 in Biphasic System with Simplified Overlay Broths.

Composition of overlay	System	24 hr Nephelos units of 1-11 dil	48 hr Nephelos units of 1-11 dil	Nephelos units of 1-11 dil	Total cells per ml in billions	Yield index	Concentration index
Dextrose-starch broth	Biphasic	28	34	64	3.3	.79	3.9
	Control	14	18	22	.84		
Idem, less starch	Biphasic	43	56	66	2.6	.57	2.9
	Control	13	14	15	.91		
" , less gelatin	Biphasic	41	49	67	3.2	.58	2.9
	Control	12	16	19	1.1		
" , less gelatin & starch	Biphasic	31	31	34	1.4	.38	1.9
	Control	10	11	12	.73		
Distilled water only	Biphasic	18	34	50	2.2		

Underlay in all cases consisted of 100 ml of complete dextrose-starch medium with 2% agar. The 125 ml of medium in control flasks had the same composition as the 25 ml of overlay in respective biphasic flasks. A water blank read 5 and a 1-11 dilution of complete broth read 11 nephelos units; turbidity data are not corrected for these blanks.

equilibrate on the shaker for 24 hours before inoculation. These biphasic flasks together with broth controls of the same composition as the overlay media then were inoculated and incubated on the shaker. Representative results of such experiments are given in Table I. More rapid multiplication was seen in all of the biphasic flasks, although subsequent studies with other bacteria have indicated that rate differences measured nephelometrically may only reflect cell size differences. By 72 hours, excellent and fairly comparable growth had occurred in all of the simplified biphasic overlay broths, representing population densities at least 2 to 4 times greater than broth controls although at some expense of total yields.

The comparison in Table I of the complete with simplified overlay media is not entirely representative, since in confirming experiments the complete medium gave the greatest density at all times of sampling. Either starch or gelatin, but not both, could be omitted from the biphasic overlay broth without appreciably affecting final population density. Remarkably, when water was added over complete dextrose-starch agar and allowed to equilibrate, the gonococcal growth attainable was only a little below that in complete medium. This lower yield partly reflects the reduced total amount of nutrient in the water/agar system. Even so, the growth attained in this clear and almost colorless menstruum was more than twice the density of that in control flasks with complex media.

Discussion and summary. The results first demonstrate the applicability and usefulness of an agar/liquid flask system to obtain gonococcal growth several times more dense than normal in a representative liquid medium. Counts as high as 5×10^9 cells per ml and turbidity similar to that normally seen with staphylococci were attained.

Second and perhaps more significant, the results illustrate a principle by which the enrichment materials normally incorporated into gonococcal media can be separated from the immediate growth environment. Under the conditions existing in a biphasic flask, even a clear water extract of the complete medium supported luxuriant growth of gonococci. Such medium simplification in principle should be applicable to other fastidious organisms.

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Overwintering of Western Equine Encephalomyelitis Virus in Experimentally Infected Garter Snakes and Transmission to Mosquitoes. (26006)

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Since viremia of long duration and high titer results from the experimental inoculation of garter snakes with Western equine encephalomyelitis (WEE) virus(1), the possibility that garter snakes serve as an overwinter reservoir of WEE virus was investigated. A hibernation cage was constructed for the purpose of holding snakes and mosquitoes under simulated natural conditions.

Materials and methods. The hibernation cage, as illustrated in Fig. 1, was composed of 3 compartments and dug into the bank of a small stream. Each compartment was filled with a mound of decayed logs and debris covered by a thick layer of rocks and soil. A layer of straw was provided as insulation. Throughout the lower part of the compartment, and leading from exposed surface to area of decayed logs, wooden ducts of various lengths provided passageways for snakes and mosquitoes. A pan of water and cotton pads wet with a 10% solution of honey were kept in each compartment as long as there was evidence of mosquito activity. Temperatures during the winter were much more severe than long time averages for the area:

Month	Minimum	Maximum	Median
Nov.	–24°F	68°F	29°F
Dec.	7	56	28
Jan.	–21	49	19
Feb.	– 9	50	28
Mar.	– 9	72	25
Apr.	25	78	44
May	25	84	51

The strain of virus used was originally isolated from *Culex tarsalis* from the state of Washington in 1957 and had undergone one chick passage and 3 suckling mouse passages.

Two species of wild garter snakes (*Thamnophis sirtalis parietalis* Say and *Thamnophis ordinoides vagrans* Baird and Girard) were inoculated intraperitoneally with 1 million suckling mouse LD_{50} in 0.05 ml virus-infected mouse brain suspension and placed in the hibernation cage. As snakes began to appear following hibernation, they were captured and held in small cages in the laboratory until May 1 when they were placed within the screened entrance of the hibernation cage. Blood was obtained from snakes by snipping their tails and tested for virus content in suckling mice. Numbers of snakes used in the study, as well as the date on which each group was placed in hibernation cage, are shown in Table I.

C. tarsalis for hibernation study were collected as larvae near Provo, Utah. They were fed as adults, either on a non-infected chick or on one that had been inoculated with virus, and were placed immediately in the hibernation cage. Numbers of mosquitoes of each group, and date they were placed in the hibernation cage, are shown in Table I. Uninfected *C. tarsalis* used to demonstrate transmission of virus from overwintering snakes to

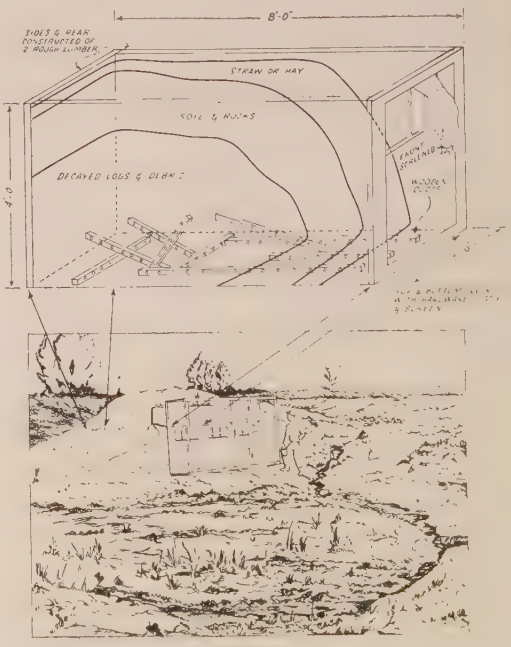

FIG. 1.

TABLE I. Mosquito and Snake Combinations in Hibernation Cage.

Compartment 1			Compartment 2			Compartment 3		
	No.	Date		No.	Date		No.	Date
Infected mosq. (total 80)	30	9/11/59	Normal mosq.	?		Infected mosq. (total 270)	155	8/28/59
	50	10/20/59					40	8/31/59
			Infected snakes (total 50)	5	9/14/59		25	9/ 1/59
Normal snakes (total 30)	20	9/11/59		10	9/18/59		50	10/20/59
	10	11/ 6/59		10	9/21/59			
				6	9/28/59	Snakes	0	
				19	11/ 6/59			

1-day-old chicks were obtained from the Communicable Disease Center, Greeley, Colo., through the courtesy of Mr. John S. Blackmore. These mosquitoes were fed on post-hibernating snakes which had viremia and held from 9 to 23 days before allowing them to feed on a non-infected 1-day-old chick.

Positive identifications of WEE virus isolated from snake bloods, mosquitoes, and chicks used to demonstrate transmission of virus were made using the mouse neutralization test(2). All virus titrations and identifications were made in suckling mice (0.05 ml, I.P.). The Reed-Muench method was used to calculate LD_{50}(3).

Results. No mosquitoes were found in any compartment following hibernation.

Twenty-five of 30 snakes were recovered from Compartment 1 between 3-23-60 and 5-9-60. No virus was detected in these snakes in a series of 3 bleedings at weekly intervals.

As shown in Table II, 26 of 50 inoculated snakes were recovered from Compartment 2. Virus was isolated from 23 of the 26 snakes with positive identification of WEE virus from 16 of the 23 snakes. Questionable virus isolations were made from 2 snakes—Nos. 6074 and 6358. No virus was detected from snake No. 5433. Virus was detected in snakes at least 70 days following recovery of snakes

TABLE II. WEE Virus Isolations from Snakes (Hibernation Study 1959-60).

Snake No.	Snake recovered from cage	Days after snake recovered on which blood tested for presence of virus	Snake died
5328	3/23/60	(0), *4*, 9	4/ 3
5326	3/24/60	*0*, 3, 8, 13	4/12
5327	3/24/60	(0), *3*, 8, 13, 19, 26, 34	5/ 6
5324	3/25/60	(0) (2), *4*, 7, 12, 18, 25, (33) (42) (53) (61) (68) (74) (81) (88) (95) (102) (109) (115)	8/ 3
5325	3/25/60	(0), *2*, 7, 12, 18, 20, 21, 25, 33, 42	5/17
5331	3/27/60	*0*, 5	4/ 3
5432	4/ 3/60	1, *3*, 9, 16, 24, 33, 44, 52, 59, 65	6/14
5433	4/ 4/60	(0) (2) (7) (15) (23) (32) (43) (51) (58) (64) (71) (78) (85) (92) (99) (105)	6/ 7
5445	4/ 4/60	1, 2, *8*, 15, 23, 32, 43, 51, 58	7/18
5446	4/ 4/60	(1), 2, 7, *15*, 23, 32, (43) (51) (58) (64) (71) (78) (85) (92) (99) (105)	6/14
5448	4/ 5/60	(0), 1, *6*, 14, 22, 31, 42, 50, 57, 63	8/ 1
5449	4/ 5/60	(0) (2), 6, 14, 22, (31), 42, 50, 57, 63, 70, (77) (84) (91) (98) (104) (111)	6/21
5451	4/ 5/60	(0), 2, *6*, 7, 14, 22, 31, 42, 50, *57*, 63, 70	7/18
5504	4/ 8/60	(0), *4*, 11, 19, 28, 39, 47, 54, 60, 67, (74) (81) (88) (95)	7/12
6018	5/ 6/60	(0) (5) (11) (19), 26, 32, 39, 46, *53*, (60)	8/ 1
6019	5/ 6/60	(0) (5), 11, 19, *26*, 32, 39, 46, 53, (60), 67, (73) (80)	6/28
6020	5/ 6/60	(0) (5) (11) (19) (26), 32, 39, 46	7/12
6021	5/ 6/60	(0) (5), 11,* 19,* 26, 32, 39, 46, *53*, 60	8/ 1
6022	5/ 6/60	(0), 5,* 11,* 19, 26, 32, 39, 46, 53, 60, 67, (73) (80)	7/14
6051	5/ 9/60	(0), 2,* 8,* (16), 23,* 29,* *36*, 43, 50, 57, (64)	6/ 1
6055	5/10/60	0, 7, 15	6/21
6074	5/12/60	(0) (7) (15) (22) (28), 35*	7/19
6075	5/12/60	(1) (8) (16) (23) (29) (36) (43), 50, 57, 64, 70	7/ 5
6357	6/ 1/60	0, 2, 6,* (13) (20) (27) (34) (41) (47)	
6358	6/ 1/60	(0) (2), 6,* 13,* (20) (27)	
6416	6/ 3/60	(0), 4, 11, (18) (25) (32) (39) (45)	

* Questionable isolation. ( ) = no virus detected. Italicized figures indicate day virus identified.

TABLE III. WEE Virus Titrations of Snake Bloods (Hibernation Study 1959-60).

Snake No.	Snake recovered from cage	Virus titrations: Day blood collected	Virus titrations: LD_{50}
5328	3/23/60	4, 9	5.8, 5.0
5326	3/24/60	3, 8, 13	2.8, 3.2, 6.3>
5327	3/24/60	8, 13, 19, 26, 34	3.9, 6.0, 4.5>, 4.3, 4.3
5324	3/25/60	7, 12, 18, 25	3.4, 2.3, <1.0, <1.0
5325	3/25/60	2, 7, 12, 18, 20, 21, 25, 42	2.4, 4.9, 4.8, 3.3, 3.4, 3.9, 3.3, 2.8
5331	2/27/60	0, 5	1.8, 2.3
5432	4/ 3/60	3, 33, 44, 52, 59, 65	5.5, 3.0, 3.3, 4.4, 2.8, 3.4
5433	4/ 4/60		
5445	4/ 4/60	1, 2, 15, 32, 43, 51, 58	2.6, 2.7, 5.3>, 5.0>, 3.5>, 2.7, 2.5
5446	4/ 4/60	15, 32	1.5, 1.5
5448	4/ 5/60	14, 31, 42, 50, 57, 63	4.8, 4.0, 2.5>, 4.3, 3.4, 2.9
5449	4/ 5/60	14, 42, 57, 63, 70	1.2, 3.5, 1.0, 2.5>, 1.0
5451	4/ 5/60	7, 14, 31, 42, 50, 57, 63, 70	4.0, 3.8, 3.2, 4.0, 3.4, 3.5, 4.3, 3.0
5504	4/ 8/60	11, 28, 39, 47, 54, 60, 67	2.2, 1.5, 2.5, 3.0, 4.0, 2.0, 1.7
6018	5/ 6/60	39, 46, 53	3.3, 2.6, 2.0
6019	5/ 6/60	11, 19, 26, 32, 39, 46, 53, 67	3.3, 2.4, 2.6, 4.5>, 1.9, 2.4, 1.3, 1.3
6020	5/ 6/60	32, 39, 46	3.3, 4.5>, 5.0>
6021	5/ 6/60	26, 32, 39, 46, 53, 60	1.5>, 3.5>, 4.1, 3.4, 3.3, 2.5
6022	5/ 6/60	26, 32, 39, 46, 53, 67	1.5>, 3.4>, 4.1, 2.6, 2.5>, 1.3
6051	5/ 9/60	36, 43, 50, 57	4.4>, 5.0, 2.8, 2.1
6055	5/10/60	7, 15	2.9, 4.4
6074	5/12/60		
6075	5/12/60	50, 64, 70	4.5, 3.4, 2.4
6357	6/ 1/60	2	2.3
6358	6/ 1/60		
6416	6/ 3/60	4, 11	4.0, 0.9

> = or greater; < = or less.

from hibernation. In many instances no virus was detected in the first blood specimen collected from the post-hibernating snakes; however blood collected after these snakes had been exposed to room temperature a few days yielded virus in most instances. In cases where the snake was bled and returned to outside conditions, there was a longer period of time during which no virus was detected.

As shown in Table III, a virus titer as high as $10^{6.3}$ LD_{50} was observed.

Virus transmissions were obtained from 4 hibernating snakes. In the case of snake No. 5324, WEE virus was isolated from snake blood, 6 of 30 mosquitoes following an extrinsic incubation period of approximately 3 weeks, and chicks that these mosquitoes fed on. WEE virus was isolated from blood of snake No. 5331, 1 mosquito that fed upon it, and the chick that the mosquito fed upon. In the case of snakes Nos. 5432 and/or 5445, the 2 snakes were placed together in the cage for mosquito feeding. Nine mosquitoes were fed on them. WEE virus was isolated from blood of both snakes, the 1 mosquito tested, and from the chick that it fed on. Virus was detected in snake bloods before and after mosquitoes fed on them in dilutions of $10^{2.3}$ to $10^{3.4}$ for snake No. 5325, $10^{1.8}$ to $10^{2.3}$ for snake No. 5331, and $10^{2.7}$ to $10^{5.5}$ for snakes Nos. 5432 and 5445.

Discussion. The failure to recover post-hibernating mosquitoes might be attributed to a number of causes, *e.g.*, the small number of mosquitoes under study and an inadequate hibernation-conditioning period following the blood meal, since the mosquitoes were placed in the cage immediately after they became engorged with blood. The unusually rapid drop in temperature from 68°F on 11-2-59 to −24°F on 11-16-59 may have caused high mosquito mortality.

Infected mosquitoes were confined with normal snakes in Compartment 1 to determine if after hibernation virus-infected mosquitoes transmitted virus to normal snakes. Since no mosquitoes survived overwinter, it was to be expected that no virus would be detected from these snakes.

Experimentally infected garter snakes were

shown to hibernate overwinter and to circulate virus in high titer and for long periods the following spring. Normal mosquitoes became infected by feeding on the emerged snakes and transmitted virus to chicks after an extrinsic incubation period of approximately 3 weeks. These data demonstrate a possible overwintering mechanism of WEE virus, but this virus has not, as yet, been isolated from garter snakes collected in the field.

Summary. Garter snakes were inoculated in September and November with Western equine encephalomyelitis (WEE) virus and were caused to hibernate under simulated natural conditions. They emerged during March, April, May and June. After varying periods when no virus was detectable in their blood, virus was detected in concentrations as high as $10^{6.3}>$ and for a period up to 70 days following emergence of snakes. Normal mosquitoes became infected by feeding on these snakes and after an extrinsic incubation period of approximately 3 weeks transmitted WEE virus to 1-day-old chicks. These data demonstrate that snakes may serve as a natural overwintering mechanism for WEE virus.

The technical assistance of Jack Cory and Edward Patzer is gratefully acknowledged.

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ECHO-4 Viruses: Improved Methods and Strain Selection for Identification and Serodiagnosis.*† (26007)

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The majority of the Enteroviruses propagated in cell culture are readily identified by standard roller tube neutralization technics (1,2). A notable exception is ECHO-4(2,3). Plaque-forming strains of ECHO-4 virus may be identified by the plaque reduction technic (2,3). The applicability of the complement fixation (CF) technic for identification of the first 13 ECHO viruses except ECHO-4 was first demonstrated by Archetti *et al.*(4) using virus antigens prepared in HeLa cells. Halonen *et al.*(5,6) later found that the fluorocarbon procedure of Gessler *et al.*(7) as applied by Manson *et al.*(8) to viral antigens grown in cell culture removed anticomplementary activity of rhesus monkey kidney cell culture (RhMKCC) fluids and greatly reduced reactions between cell culture antigens and antibodies thereto, factors otherwise posing problems in interpretation of tests used for viral identification. This report, although limited to studies on ECHO-4 virus, describes a further modification of the CF test which virtually eliminates all antibody reactions with cell-culture antigens.

Another problem in diagnostic work involving ECHO-4 infections is the general inability to demonstrate neutralizing antibodies in the convalescent serum of the patient. A strain of virus is described which has been found to be readily neutralizable in tube culture tests.

Materials and methods. 1. Cell culture. Trypsinized RhMKCC were grown in 0.5% lactalbumin hydrolysate in Hanks' (LAH) balanced salt solution (BSS) containing 2% calf serum and maintained in lactalbumin

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hydrolysate in Earle's BSS (LAE) containing 0.5% calf serum. Primary cell cultures of hamster, guinea pig and cat kidney were grown and maintained in similar media with only minor modifications of serum concentrations. Monolayer cell cultures of a human fibroblast-like continuous cell line, Detroit-504§ (Det.-504)(9), grown in Eagle's Basal Medium (EBM) supplemented with 20% pooled human serum. Primary cell cultures of human amnion and continuous cell cultures of bovine kidney,‖ HeLa (Gey),‖ H.Ep. #2¶ and cynomolgus monkey heart¶ were grown in EBM supplemented with 10% calf serum. *2. Viruses.* Prototype ECHO-4 virus, strain Pesascek, was obtained from Dr. J. L. Melnick of Houston, Texas. Five ECHO-4 strains (2219, 2212, 2235, 3112, 5705) were isolated in this laboratory from patients ill with paralytic disease or aseptic meningitis (10). Two ECHO-4 strains (Shropshire and Gregory) were isolated by Dr. David T. Karzon of Buffalo, N. Y. and supplied by him. Dr. Karzon also supplied DuToit strain isolated by Dr. Malherbe in South Africa. *3. Serology.* Serum neutralizing antibody (NA) titration methods have been described(10). Fifty per cent endpoints were calculated by the method of Reed and Muench(11). CF tests were performed in 13 x 100 mm tubes using 0.2 ml of each reagent. Antigen (virus) and antiserum plus 2 full units of guinea pig complement were incubated 1 hour at 37°C. Sensitized sheep red blood cells were then added and the test incubated another hour at 37°C. Endpoints represented the highest 2-fold dilution showing no more than 50% hemolysis as determined by visual comparison with hemolytic standards. Viral CF antigens were undiluted cell culture fluids clarified by centrifugation at 2,000 rpm for 10 minutes and extracted with fluorocarbon once according to the method of Manson *et al.*(8). *4. Antisera.* Monkey ECHO-4 antiserum prepared by Dr. Herbert A. Wenner(2) was supplied through the courtesy of The National Foundation. Additional ECHO-4 antisera were produced, as follows, in guinea pigs and monkeys in this laboratory. Guinea pigs received 6 intramuscular injections of 0.5 ml of fluorocarbon-treated virus concentrate emulsified in 0.5 ml of Arlacel-Bayol F adjuvant at approximately 10 day intervals. Monkeys were immunized by a similar regime which employed 2.0 ml of purified virus concentrate and 2.0 ml of adjuvant per injection. The procedure for concentration of fluorocarbon-treated enterovirus antigens for immunization of animals has been described(12). Monkey and guinea pig antisera used in CF tests were inactivated for 30 min at 60°C and 56°C respectively. Certain antisera were adsorbed by mixing equal volumes of undilute or 1:5 dilution of antisera and 10% suspension of trypsinized RhMK cells, incubating 1 hour at 37°C and then centrifuging at 2,000 rpm for 30 minutes at 4°C.

Results. 1. Tube neutralization identification tests. Four ECHO-4 antisera were tested against 9 strains of ECHO-4 virus. Results (Table I) represent reciprocals of 50% serum dilution endpoints obtained at the time the final $TCID_{50}$ endpoint in the control virus titrations was first reached. Three strains, 3112, 5705 and DuToit, were neutralized effectively by all 4 antisera; 2 strains, Shropshire and Gregory, were not significantly neutralized by any antiserum. None of the 3 antisera produced with prototype strain Pesascek (columns 1, 3 and 4) significantly neutralized strains 2212, 2219 and 2235. A monkey antiserum to strain 2219 (column 2), a non-plaque forming virus, neutralized these 3 strains and strain Pesascek to a slight degree, dilutions 1:40 to 1:120. In all but 2 instances there was a further significant drop (>4-fold) in NA titer of all antisera after the $TCID_{50}$ endpoint was noted initially, *i.e.*, when no further cytopathogenic effect (CPE) occurred in the virus titration controls. The exceptions occurred with DuToit and 5705 strains. Strain DuToit was solidly neutralized with all 4 antisera; slight decreases ($\leq$4-fold) occurred with strain 5705. *2. CF identification tests.* Two major problems which arose during early CF studies with ECHO viruses were reactions of antisera with

§ Cell line obtained from Dr. Cyril S. Stulberg, Detroit, Mich.

‖ Cell line obtained from Microbiological Assoc., Washington, D.C.

¶ *idem* from Dr. Jonas Salk, Pittsburgh, Pa.

TABLE I. Neutralizing Antibody Titers of 4 ECHO-4 Antisera against 9 Strains of ECHO-4.

ECHO-4 virus		ECHO-4 antisera: Strain; cell source; animal source			
Strain	$TCID_{50}$	Pesascek* RhMKCC Monkey†	2219 RhMKCC Monkey	Pesascek* RhMKCC G. pig	Pesascek* Det.-504 G. pig
Pesascek*	100	*80*‡	120	*60*	*30*
2219	100	5	*60*	10	10
2212	100	20	40	10	5
2235	32	20	80	10	10
Shropshire	100	<5	<5	5	<5
Gregory	320	5	5	7.5	7.5
3112	200	240	240	160	80
5705	200	5120	5120	640	320
DuToit	200	>640	640	320	320

* Prototype ECHO-4 virus. † Wenner antiserum. ‡ Reciprocal of 50% serum dilution endpoint.

RhMKCC = Rhesus monkey kidney cell culture; Det.-504 = Detroit-504 cell culture; G. pig = guinea pig.

RhMKCC antigens and anti-complementary activity of RhMKCC virus antigens. The latter problem was minimized when EBM without serum was employed as the medium for virus growth followed by a single fluorocarbon extraction of the virus cell culture fluids, as described by Halonen *et al.*(6). This treatment, however, does not remove all cell culture antigens. Thus, low dilutions of antisera produced with virus grown in RhMKCC will still react with virus grown in homologous cell culture. Adsorption of these antisera with trypsinized RhMKCC generally removes these non-viral reactions.

A more desirable means to eliminate cell culture antibody reactions would be to propagate virus for CF antigen in a cell culture heterologous to that used to grow virus for immunization, as had been done with certain ECHO viruses in HeLa cells by Archetti *et al.* (4). Unfortunately, ECHO-4 does not propagate in HeLa cells. Nonetheless, with this premise in mind, attempts were made to adapt Pesascek and 2219 strains to primary cell cultures of hamster, guinea pig and cat kidney, human amnion and to continuous cell lines of bovine kidney, HeLa (Gey), H.Ep. #2, and cynomologus monkey heart, all with no success. During these studies, Stulberg *et al.*(9) reported propagation of the first 16 ECHO viruses, including ECHO-4, in a human fibroblast-like cell line. A similar line, Det.-504 was obtained from Dr. Stulberg. Two ECHO-4 strains, Pesascek and 2219, were readily adapted to Det.-504 in this laboratory. After 6 passages in Det.-504, a titer of $10^{5.7}$/ml was obtained with the Pesascek strain. A pool of virus was prepared, extracted twice with fluorocarbon, concentrated 10-fold and used to immunize 8 guinea pigs and 1 monkey. Tube NA tests with the resultant guinea pig antisera are shown in Table I; similar low levels of NA were obtained with the monkey antiserum (not shown). These 2 antisera from Det.-504 cultures and 4 other ECHO-4 antisera produced with RhMKCC grown virus were titrated for CF antibodies against antigens of 9 strains of ECHO-4 virus. Results are shown in Table II. Titers of Wenner's monkey antiserum, strain Pesascek (column 1) ranged from 1:160 (strain DuToit and Shropshire) to 1:2048 (strain 2212) whereas a similarly prepared antiserum to strain 2219 (column 2) ranged from 1:512 to 1:2048. Titers of antisera produced in guinea pigs immunized with Pesascek (column 3) and 2219 (column 4) viruses grown in RhMKCC ranged from 1:120 to 1:2560 and from 1:80 to 1:2560 respectively. Guinea pig and monkey antisera produced with Pesascek virus grown in Det.-504 cells (columns 5 and 6 respectively) showed somewhat lower titer ranges of 1:24 to 1:1024 and 1:32 to 1:1024 respectively. Strain 2212 showed strongest reactions (1:1024 to 1:2560) with all antisera. In contrast, strains Shropshire and DuToit produced reactions ranging from 1:24 to 1:640 and

TABLE II. Complement-Fixing Titers of 6 ECHO-4 Antisera against 9 Strains of ECHO-4.

ECHO virus*		ECHO-4 antisera: Virus strain; cell source; animal source					
		Pesascek† RhMKCC‡	2219 RhMKCC‡	Pesascek† RhMKCC‡	2219 RhMKCC‡	Pesascek† Det.-504	Pesascek† Det.-504
Type	Strain	Monkey§	Monkey	G. pig	G. pig	G. pig	G. pig
4	Pesascek†	1024‖	1024	1280	640	512	256
4	2219	1024	1024	640	640	128	512
4	2212	2048	2048	2560	2560	1024	1024
4	2235	512	512	640	1280	256	128
4	Shropshire	160	640	120	80	24	64
4	Gregory	640	1920	960	640	128	128
4	3112	1024	1024	320	640	128	128
4	5705	1024	2048	960	320	256	256
4	DuToit	160	1280	320	120	48	32
12	Travis†	<10	<10	<10	<10	<4	<4
	RhMKCC¶	<10	<10	<10	<10	<4	<4
	RhMKCC *vs* unadsorbed antiserum	20	40	40	160		

* Virus antigens grown in Eagle's Basal Medium, extracted once with fluorocarbon.
† Prototype virus strain.
‡ Antiserum adsorbed with trypsinized RhMKCC.
§ Wenner's ECHO-4 antiserum.
‖ Represents reciprocal of 50% serum dilution endpoint.
¶ Prepared from twice frozen and thawed RhMKCC, clarified by centrifugation only.

from 1:32 to 1:1280 respectively. No cross-reaction occurred with ECHO-12 strain Travis and with RhMKCC control fluids at 1:10 dilution of 4 antisera produced with virus grown in RhMKCC when adsorbed with RhMKCC. Prior to adsorption these 4 antisera showed cross reactions with RhMKCC which ranged from 1:20 to 1:160. In contrast, 2 antisera produced with virus grown in Det.-504, even though not adsorbed, did not cross react with RhMKCC at 1:4 dilution.

Discussion. It has been our experience that the tube neutralization technic is an unreliable method for serologic identification of ECHO-4 viruses. Apparently other investigators have encountered similar difficulties (2,3). The lack of a readily neutralizable strain of ECHO-4 has presented serious problems in titration of serum NA to ECHO-4 viruses. In this laboratory it had been found that NA in patients' sera could be detected best with the patients' homologous virus (10). Recently a strain (DuToit) was reported available by Barron and Karzon (3) which was readily neutralized in tube tests. These investigators have employed the DuToit strain to titer NA in sera of persons infected with other ECHO-4 strains. As shown herein, the DuToit and 5705 strains are nearly equally neutralizable in tube tests. It would appear that both strains may be used to titrate NA.

To our knowledge, no ECHO-4 strain has been isolated which produces in immunized laboratory animals an antiserum that effectively neutralizes in the tube test the majority of strains isolated. Thus, the search is still on for a strain of ECHO-4 which will stimulate production of NA effective against all strains of ECHO-4 in the tube neutralization test.

Lacking a simple tube neutralization test, the most reliable method for laboratory identification of ECHO-4 viruses has been the CF test (2,4,5,6). Although in the studies reported here 2 strains, Shropshire and DuToit, did not react with the higher dilutions of all antisera, specific identification of these strains and the 7 other strains tested is assured by the lack of cross-reactions at even a 1:4 dilution with controls. Reactions between RhMKCC antigens and antibodies directed thereto, which were stimulated by immunization of animals with virus grown in RhMKCC, were obviated by fluorocarbon treatment of virus CF antigens and adsorption of the antisera with RhMKCC. This latter procedure was of absolute necessity with those antisera produced in guinea pigs

since CF titers of 1:40 to 1:160 to RhMKCC had been noted in these same antisera prior to adsorption. Habel *et al.*(13) have produced CF titers of 1:3840 to both HeLa and RhMKCC when rabbits were immunized with ECHO-4 grown in RhMKCC that had not been extracted with fluorocarbon. In the work reported here there can be no doubt concerning the specificity of the CF reactions obtained with the 2 antisera produced with Pesascek virus grown in Det.-504 cells since a 1:4 dilution of both antisera, even though not adsorbed with RhMKCC, did not react with either undiluted RhMKCC fluids clarified by centrifugation or with other ECHO viruses grown in RhMKCC and extracted once with fluorocarbon. This specificity may be due to lack of antigenic relationship between RhMKCC and Det.-504 or to removal of sufficient cell culture antigens by fluorocarbon treatment prior to animal immunization, and use of the guinea pig instead of the rabbit for source of antiserum.

Recently Sweet and Hilleman(14) reported that a very high percentage of RhMKCC contains high titers of a virus cytopathogenic for *Cercopithecus aethiops* (African green) MKCC. The presence of this agent in RhMKCC grown virus may account in part for cross-reactions obtained with CF virus grown in RhMKCC and antisera produced with similarly propagated heterologous virus. The lack of cross-reactions in studies reported here indicates that adsorption of antisera with RhMKCC apparently containing large amounts of this virus may remove much of the antibody for this agent.

The obvious advantage of employing ECHO-4 virus grown in Det.-504 for animal immunization and virus grown in RhMKCC for CF antigen is that adsorption of antisera is not necessary to assure reaction specificity even with virus antigens of low antigenicity. Another advantage of this system is the use of the guinea pig to replace the more expensive monkey as source of immune serum for the CF identification test. In addition, these studies have demonstrated that the ECHO-4 antibody produced in guinea pigs immunized with virus grown in Det.-504 is specific and does not react with the RhMKCC antigens remaining in virus-containing fluids after a single fluorocarbon treatment. Although no attempt has been made in this laboratory to extend this particular "heterologous CF" system to identification of other ECHO viruses, it seems reasonable to expect that extension is possible since Stulberg *et al.*(9) have demonstrated that 15 other ECHO virus types tested will grow in a similar human fibroblast-like cell line.

Summary. These studies have shown that the tube neutralization identification test for ECHO-4 viruses is unreliable and that the most reliable identification method at present is the CF test. It has been demonstrated that immunization of guinea pigs and monkeys with ECHO-4 virus grown in Det.-504 human fibroblast-like cells produces an antiserum free of antibody which will react with RhMKCC antigens, thereby eliminating cell culture cross-reactions and yielding a highly specific diagnostic reaction even with virus antigens of low antigenicity. The ability of ECHO-4 antibodies to readily neutralize DuToit strain as reported by Barron and Karzon(3) has been confirmed and our strain 5705 was found to have similar properties. As suggested by these investigators, DuToit-like strains should probably be employed to detect NA in human patients' sera.

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Hematologic Response of Monkeys to X-irradiation.* (26008)

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Previous reports from this institution have described pathology(1) and antibody response(2) in monkeys following total body x-irradiation. As part of the over-all project, the effect of irradiation on the peripheral blood picture was also studied. Most investigations into the effect of total body x-irradiation on cellular elements of the blood have been conducted in subprimate species. In the relatively few studies(3-5) concerned with monkeys, varying dose levels and schedules have been employed. This report describes hematologic response of monkeys following a single total-body exposure to 450 r.

Materials and methods. A total of 37 young adult monkeys (1½-3 years old, *Macaca mulatta*) received total body irradiation with 450 r by a previously described method (1). Group II (18 monkeys) was irradiated 6 months after Group I (19 monkeys). Seven control monkeys were handled in an identical manner, but received no irradiation. Blood counts done from ear lobes were obtained 4-5 times over a 2-week base-line period, and then at 2, 4, 10 and 12 days after irradiation; subsequent counts were then made twice weekly for 2 months and weekly up to 6 months. Hemoglobin content was determined with a Leitz-Rouy Photrometer. Differential counts were made of 200 cells stained with Wright's stain.

Results. The effect of irradiation in the 37 monkeys was first reflected by the leukocytes, which were depressed significantly at 2 days, markedly at 4 days, and maximally between 10-17 days (Fig. 1). At the latter intervals, mean leukocyte count was only 5% of base-line level. Beginning recovery of leukocytes, noted at 19 days, was followed by a marked leukocytosis in 17 of 37 monkeys between 4 and 7 weeks post-irradiation. Recovery occurred at variable rates in the remaining animals, the low mean leukocyte count at 60 days reflecting the fact that half the animals had not attained base-line levels. By 3 months mean leukocyte count had returned to pre-irradiation level.

The effect of irradiation on erythrocytes (Fig. 1) was noted later and to a less marked degree than the effect on leukocytes. Erythrocytes were not significantly decreased at 2 and 4 days, and lowest levels were not reached until 17-19 days post-irradiation, at which time mean count was about half pre-irradiation value. Beginning recovery at about 4 weeks was followed by a gradual increase to base-line level at about 3½ months. In general, depression and recovery of hemoglobin paralleled that for erythrocytes. A gradual decline in hemoglobin concentration was noted in control, non-irradiated monkeys, but all other elements remained stable during the observation period.

The effect of irradiation on differential leukocyte count in 18 monkeys (Group II) is presented in Table I, with data on erythrocytes included for comparison purposes. Lymphocytes showed early reflection of irradiation with a rapid decline noted by the second day and a mean level of only 9% of base-line at 12 days. Gradual recovery began about the 20th day, with return to pre-

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TABLE I. Mean Erythrocyte and Leukocyte Counts in 18 Monkeys (Group II) after a Single Dose of 450 r.

Days after irrad.	Erythrocytes		Total leukocytes		Neutrophiles		Lympho-cytes		Eosinophiles		Baso-philes	Mono-cytes
	$\times 10^6$	S.D.*	$\times 10^3$	S.D.	$\times 10^3$	S.D.	$\times 10^3$	S.D.	$\times 10^3$	S.D.	$\times 10^3$	
0	5.2	.7	14.8	5.2	6.5	4.1	7.0	1.8	.8	.5	.06	.48
2	4.9	.8	5.5	2.6	4.4	2.6	1.0	.5	.1	.2	.02	.03
4	4.7	.8	4.4	2.2	3.5	2.1	.8	.3	.1	.1	.01	.02
10	4.1	.8	1.0	.5	.3	.2	.7	.4	0	—	.00	.00
12	3.7	.9	.9	.6	.2	.2	.7	.6	0	—	.00	.00
17	3.2	1.1	1.4	.9	.5	.8	.8	.7	0	—	.01	.00
19	3.2	1.0	3.8	3.6	1.9	2.3	1.7	1.7	0	—	.01	.05
24	3.6	1.0	14.4	9.6	10.0	8.1	4.3	1.9	.1	.1	.00	.03
26	3.6	.9	17.7	12.0	12.9	10.0	4.7	2.4	.1	.1	.04	.10
31	4.1	1.0	16.6	7.5	12.5	7.2	3.1	1.2	.8	1.4	.08	.16
33	4.2	.6	15.7	6.8	10.2	6.5	3.9	1.4	1.4	2.3	.14	.05
38	4.4	.6	22.4	8.7	13.1	5.8	3.7	1.4	5.3	7.6	.11	.24
40	4.4	.7	26.3	8.3	13.4	7.1	4.6	2.0	7.8	6.9	.32	.20
45	4.7	.8	20.4	7.4	11.0	4.7	4.0	1.9	5.1	4.3	.09	.21
47	4.7	.6	17.2	6.3	10.2	4.3	3.1	1.2	3.7	4.1	.03	.15
52	4.9	.6	14.7	5.6	8.6	4.0	3.8	1.7	2.1	2.0	.06	.15
54	4.6	.6	11.0	2.5	5.1	2.1	3.8	1.2	2.1	1.5	.00	.03
60	5.1	.7	12.6	4.5	7.1	5.4	4.2	2.3	1.1	1.0	.04	.15
65	4.9	.5	11.8	2.6	5.6	3.0	4.7	2.0	1.3	.9	.00	.22
75	5.1	.5	13.8	9.1	5.7	4.6	6.4	3.2	1.5	1.1	.04	.16

* Stand. dev.

irradiation levels at about 2½ months. Neutrophiles were affected less promptly but more severely with the greatest depression to 3% of base-line level noted at 12 days. Recovery of neutrophiles paralleled the pattern described above in reference to total leukocytes (Fig. 1). Ten of 18 monkeys exhibited an increase in neutrophiles to at least twice base-line level between 24 and 47 days post-irradiation. Eosinophiles were essentially absent from the 4th to 26th post-irradiation days. Beginning recovery, noted at 31 days, was followed by definite eosinophilia in 12 of 18 monkeys. In monkeys not showing eosinophilia counts rose erratically to base-line level at about 2 months. Monocytes and basophiles were depressed to lowest levels from 10-17 days post-irradiation. Rate of recovery was variable and pre-irradiation values were reached at approximately 2 months.

Fig. 1 and Table I present data on both survivors and non-survivors since no differences were observed. Eleven of 37 monkeys died during the course of the study and all showed typical signs of radiation sickness. Three exhibited definite signs of recovery, hematologically, before death. One had a marked leukocytosis (45,000) just prior to death at 31 days post-irradiation, and in one monkey, total leukocyte count remained at very low levels until death at 96 days. This monkey was the only one in which hematologic findings were not similar to those observed in survivors.

Discussion. Results of the present study are much like those obtained by other workers(3-5), but minor differences, possibly dose or dose-rate dependent are evident. The pattern of the late eosinophilia seen in our monkeys after 450 r (23 r per minute) was similar to that described by Eldred(3) in monkeys

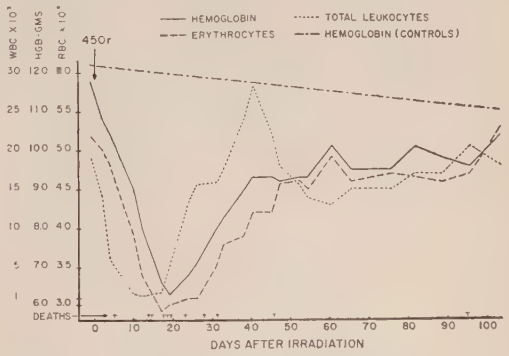

FIG. 1. Mean leukocyte, erythrocyte, and hemoglobin values in 37 monkeys after a single dose of 450 r.

given 550-600 r (13.7 r per minute) in a single session. The depression of all cellular elements was more severe in our series than in that of Riopelle, Ades and Morgan(4) who irradiated 4 monkeys with 350 r (17 r per minute). Our results show certain marked similarities to those of Haigh and Paterson (5) who irradiated 50 monkeys with 260 r, 28 with 500-550 r, and 11 with 600 r (all at 3 r per minute). The effect of 450 r on erythrocytes, eosinophiles and lymphocytes in our monkeys was much like that observed after their intermediate dose, 500-550 r. The response of neutrophiles was also similar except that the secondary elevation appeared to begin earlier in our study. Haigh and Paterson(5) observed peak neutrophile counts on the 26th and 41st days after 500-550 r, and attributed this phenomenon to "a consistent and exact duration of inhibition of precursor cells, related to dose." An almost identical picture was observed in our monkeys in that peak neutrophile and total leukocyte counts were seen consistently on the 26th and 40th days after 450 r (Table I). The secondary elevation of monocytes to supernormal levels observed by Haigh and Paterson(5) was not noted in our series.

Summary. Total body x-irradiation of monkeys with 450 r resulted in significant depression of leukocytes as early as second day with maximal effect noted between 10-17 days. Erythrocytes were affected at a later interval with maximum depression at 17-19 days post-irradiation. Recovery of leukocytes and erythrocytes to normal levels was observed between 3-3½ months.

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Electrophoretic Fractionation of B_{12}-Binders in Gastric Juice from Patients with Pernicious Anemia and from Controls.* (26009)

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The intrinsic factor (I.F.) of gastric juice has not been chemically isolated. There is some evidence that it may be associated with B_{12}-binding(1-4), although several B_{12}-binders without I.F. activity have been isolated (5-8), as have substances with high I.F. activity and a remarkably low B_{12}-binding capacity(9,10). However, whether or not I.F. actually binds Vit. B_{12}, it seems to be intimately associated with a B_{12}-binder in gastric juice(4), which may accordingly be used to trace I.F. The present study compares B_{12}-binders in gastric juice from pernicious anemia patients with those from controls.

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Methods. Human gastric juice was collected by a previously described method, with *in vivo* neutralization of the acid juice to prevent peptic autodigestion(11). Precautions were taken to avoid extragastric contaminants. The fasting secretion as well as the histamine, carbamylcholine chloride, and insulin-stimulated secretions were investigated. Within 3 hours after collection, $B_{12}Co^{60}$ was added to the juice. In some cases total B_{12}-binding capacity of gastric juice was estimated by a dialysis technic(12). In these cases, amount of Vit. B_{12} added for electrophoresis is expressed as percent of total binding capacity (Tables I, II). Following concentration and ultrafiltration through collodion membrane for about 24 hours at +4°C,

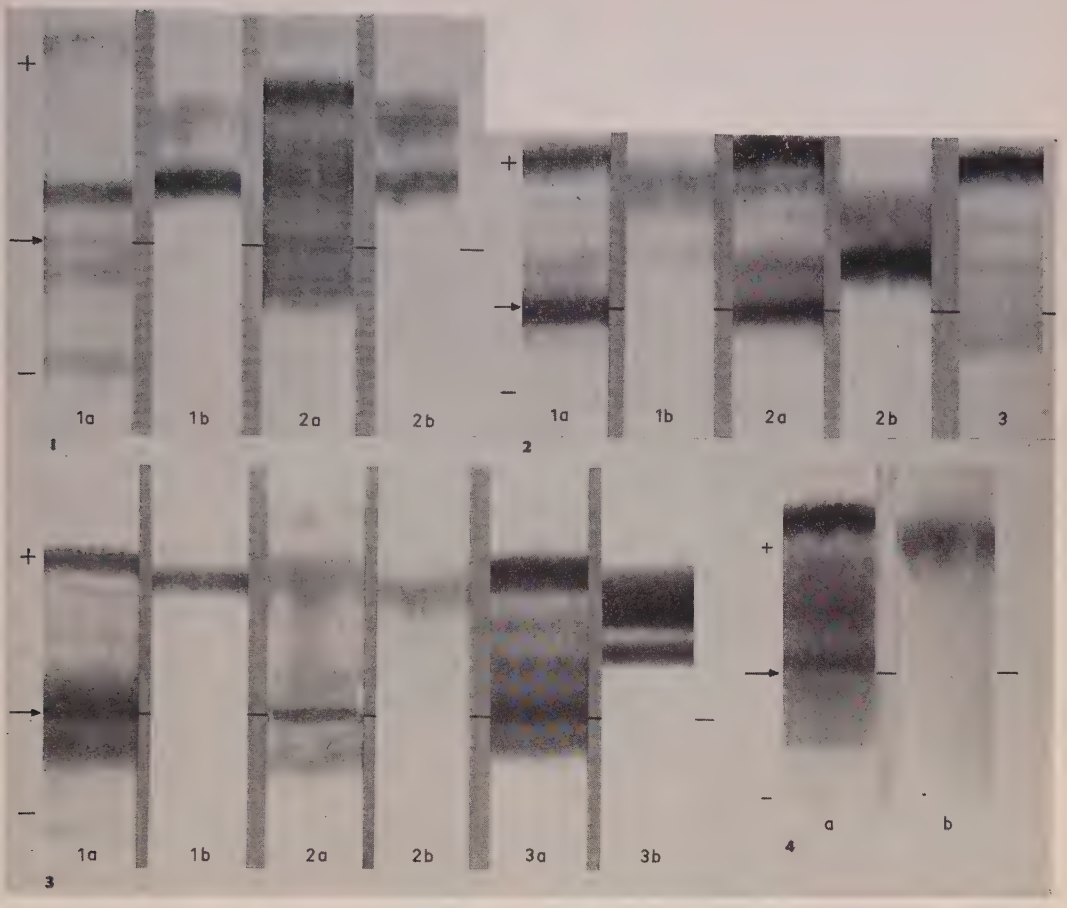

FIG. 1. Acid gastric juice. 1a. Gastric juice of pH 3, not neutralized. Paper electrophoretic strip. Amido black stain. 1b. Autoradiogram of 1a. Two B_{12}-binders are present. 2a. Same individual. Paper electrophoretic strip of gastric juice neutralized in stomach to pH 7.2. Amido black stain. 2b. Autoradiogram of 2a. Two B_{12}-binders are present.

FIG. 2. Gastric juice from patient with histamine-fast achlorhydria and normal Schilling test (Table I, case 7). 1a. Fasting secretion. Paper electrophoretic strip. Amido black stain. 1b. Autoradiogram of 1a. Two B_{12}-binders are present, but slower component is only just visible. 2a. Carbamylcholine chloride stimulation. Paper electrophoretic strip of gastric juice. Amido black stain. 2b. Autoradiogram of 2a. Compared with fasting secretion, there is a relative increase of slower component. 3. Paper electrophoretic strip of diluted serum to show localization of albumin. Amido black stain.

FIG. 3. Gastric juice from patient with histamine-fast achlorhydria and normal Schilling test (Table I, case 8). 1a. Fasting secretion. Paper electrophoretic strip. Amido black stain. 1b. Autoradiogram of 1a. Slower migrating B_{12}-binder is absent. 2a. Same juice as 1a and 1b, incubated with crystalline pepsin at pH 2 at 37°C for 30 min. before addition of vit B_{12}. Paper electrophoretic strip. Amido black stain. 2b. Autoradiogram of 2a showing no appreciable change of B_{12}-binder. 3a. Carbamylcholine chloride stimulation. Paper electrophoretic strip of gastric juice. Amido black stain. 3b. Autoradiogram of 3a. Two B_{12}-binders are present.

FIG. 4. Gastric juice from patient with pernicious anemia (Table II, case 5). a. Carbamylcholine Cl stimulation. Paper electrophoretic strip of gastric juice. Amido black stain. b. Autoradiogram of a. Slower migrating B_{12}-binder is absent.

the juice was subjected to paper electrophoresis in borate buffer of pH 9.0 and ionic strength of 0.12(13). Kodak Kodirex film was used for autoradiography, with exposure times of 4 to 7 days. The paper strips were placed on the film unstained, since bound B_{12} is released on heat-drying of the strips(1,14) and is then washed out during the staining procedure. Since it has been shown that peptic digestion has different effects on various

TABLE I. B_{12}-Binders in Gastric Juice from Achlorhydria Patients with No Evidence of Pernicious Anemia.

Age	Sex	Histamine-fast achlorhydria	Mode of stimulation of gastric glands	Added B_{12} in % of total binding capacity of gastric juice (%)	Slow migrating B_{12}-binder (? I.F.)	Fast migrating B_{12}-binder	Schilling test; 48 hr urine excretion (%)*
58	♂	+	Fasting		++	+	33
53	♀	+	Fasting		+	++	20
			Carbamylcholine	32	++	+	
71	♀	+	Carbamylcholine	21	+	+	28
55	♂	+	Fasting	2	+	++	50
			Carbamylcholine	4	+	++	
63	♀	†	Carbamylcholine		+	+	34
49	♂	†	Insulin		+	+	17
59	♀	+	Fasting		(+)	+	28
			Carbamylcholine		+	+	
40	♂	+	Histamine	31	—	+	29
			Carbamylcholine	18	+	+	
44	♂	+	Fasting		—	+	28
			Carbamylcholine		+	+	
29	♀	†	Fasting		—	+	39
			Carbamylcholine		+	++	
50	♀	+	Fasting	30	(+)	+	10%
			Carbamylcholine	30	(+)	+	On control 14% With I.F. 51%
71	♂	+	Carbamylcholine		—	+	11% With I.F. 24%

* Normal, $\geq 16\%$; normal-borderline, 11-15%; pathologic, $\leq 10\%$.
† Histamine test not performed.

B_{12}-binders (12), its effect on their electrophoretic mobility was studied. For the B_{12} absorption test a modified Schilling technic with carbamylcholine chloride stimulation was employed (15). *Material.* 1. Fifteen control subjects with hydrochloric acid present in the gastric juice. 2. Twelve patients with gastric achlorhydria (present in 9 even after histamine stimulation), but without evidence of pernicious anemia. The Schilling test was carried out in all twelve. 3. Nine patients with clinically established pernicious anemia. Five of these patients were subjected to the Schilling test with or without I.F.

Results. Two B_{12}-binders were found on electrophoretic fractionation of gastric juice from controls neutralized *in vivo* (Fig. 1). The slower and anodically migrating B_{12}-binder was always present. The faster migrating binder could not be demonstrated in 3 of the 15 cases, and even in the others most of the vitamin was generally bound to the slower component. However, less B_{12} was added than was required to saturate the binders, and it remains to be determined whether the 2 binders have differing affinities for Vit. B_{12}.

Two B_{12}-binders were found also in gastric juice from patients with *achlorhydria* and *normal Schilling test* (Table I, Fig. 2), but here relatively less of the Vit. B_{12} seemed to be bound to the slow component. In 3 cases the slower component was absent in the fasting secretion or in that following histamine stimulation, but appeared following parasympathetic stimulation (carbamylcholine chloride) (Fig. 3). A relative increase of binding to the slower component was noted in other patients following carbamylcholine chloride stimulation (Fig. 2). These observations are of interest in view of the known fact that parasympathetic stimulation augments secretion of I.F. (16). The slower component appeared to be altogether absent in one case. This person had a borderline value in the Schilling test and a normal one in the Schilling

TABLE II. B_{12}-Binders in Gastric Juice from Pernicious Anemia Patients.

Age	Sex	Mode of stimulation of gastric glands	Added B_{12} in % of total binding capacity of gastric juice (%)	Slow migrating B_{12}-binder (? I.F.)	Fast migrating B_{12}-binder	Schilling test; 48 hr urine excretion (%)* Without I.F.	With I.F.
42	♀	Fasting	46	—	+	2	30
37	♀	Fasting	41	—	+	1	19
		Carbamylcholine	55	—	+		†
57	♂	Carbamylcholine	75	—	+	.5	‡
67	♂	"	13	—	+	0	
36	♂	"		—	+	1.5	25
50	♂	"		—	+		
82	♀	"		—	+		
71	♂	"		—	+		
72	♂	Fasting		—	+		

* Normal, $\geq 16\%$; normal-borderline, 11-15%; pathologic, $\leq 10\%$.
† 13% with gastric juice from case 4, Table I.
‡ 8% " " " " case 2, Table I.

test with I.F. (Table I, case 12).

In the gastric juice from patients with *pernicious anemia* the slower migrating B_{12}-binder was invariably absent, whereas the faster component was, in all cases, present (Table II, Fig. 4).

Discussion. In normal gastric juice, two B_{12}-binders have been demonstrated electrophoretically earlier but only the slower migrating one seemed to have I.F. activity(3). The present study shows that this B_{12}-binder is absent in gastric juice from pernicious anemia patients. The faster B_{12}-binder migrates somewhat slower than albumin (Fig. 2). It seems to have the same electrophoretic mobility as the main serum B_{12}-binder in the α_1-globulin fraction(17). Gräsbeck's(3) suggestion that this component of gastric juice is a peptic breakdown product is inconsistent with its invariable presence in achlorhydria. Furthermore, peptic digestion did not seem to make significant alteration in electrophoretic pattern of the B_{12}-binders according to some preliminary investigations (Fig. 1 and 3). The method described may well be serviceable for *in vitro* assay of intrinsic factor. It could be of value especially in early diagnosis of pernicious anemia. Deficiency of the slower migrating B_{12}-binder may indicate that the patient will eventually develop pernicious anemia.

Summary. Radiovitamin B_{12} was added *in vitro* to human gastric juice. Paper electrophoresis was done with ultrafiltrated juice, and the strips autoradiographed. Two B_{12}-binders were found. The slower, anodically migrating B_{12}-binder was always present both in normal gastric juice and in gastric juice from patients with histamine-fast achlorhydria and normal Schilling test. It was invariably absent in pernicious anemia, where only the faster component was found. In some achlorhydria cases with normal Schilling test the slower migrating B_{12}-binder was identified following parasympathetic stimulation, but absent in fasting secretion or in that following histamine stimulation. The investigation supports the view that the slower migrating B_{12}-binder observed in electrophoresis of gastric juice is related to intrinsic factor. It is suggested that the method may be of diagnostic value.

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Artificial Tumors in Dog Bronchus and Their Implications in Production of Experimental Emphysema.* (26010)

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Early diagnosis of pulmonary tumors by X-ray studies is difficult, and endobronchial lesions often go undetected until they occlude the bronchial lumen with resultant distal atelectasis. The original purpose of this study was to produce lesions in experimental animals that simulate endobronchial neoplasms and to demonstrate these lesions by a bronchographic technic recently described(1). While the bronchographic study was being carried out, it was observed that with increasing time after the lesions were made, some of these animals showed evidence of chronic, progressive pulmonary disease suggesting emphysema. This report presents results of this initial experiment.

Materials and methods. Two methods of producing intrabronchial lesions in animals were tried, (1) ligation of individual bronchi to produce incomplete stenosis, and (2) injection of a plastic material to produce an endobronchial mass which would partially obstruct a bronchus. The first method was ineffective in that the incomplete obstruction produced by constricting ligatures did not result in a demonstrable lesion and could not be effectively studied by X-ray. This method was discarded and the second procedure investigated. To find a satisfactory plastic substance, studies were carried out on various materials. Preliminary observations were made with 4 different types of liquid plastics, which were injected subcutaneously into hamsters. Three of these plastics caused severe inflammatory reactions, followed by varying degrees of necrosis at site of injection. The fourth material, *i.e.,* an acrylic resin emulsion called Rhoplex AC-33, (product of Rohm & Haas Co. of Philadelphia) produced little inflammation or necrosis and apparently acted as a bland foreign body after hardening *in situ*. This material is a milky liquid containing 46% solids and is miscible with water. The Rhoplex was thickened by addition of a sodium polyacrylate, Acrysol G.S., which is supplied by the same company at 12-13% solids in water solution. The latter is extremely viscous and may be handled more easily if diluted with an equal volume of distilled water. When 10 ml of the resulting mixture is added to 20 ml of Rhoplex AC-33, a thick creamy emulsion is obtained, which flows readily through a 25 gauge needle. This preparation may be easily maintained for long periods at room temperature if kept covered. Large dogs were utilized for production of the bronchial lesions. During thoracotomy, a lobar or segmental bronchus was prepared by clearing adjacent connective tissues, and 0.5 to 1.0 ml of the thickened acrylic resin emulsion was injected transbronchially and deposited at the immediate sub-mucosal level. Care was taken not to puncture bronchial mucosa and enter the bronchial lumen. This can read-

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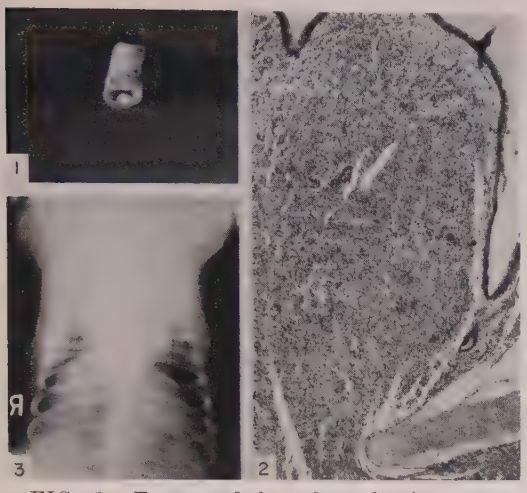

FIG. 1. Transected bronchus showing tumor. × ⅓.
FIG. 2. Cross section of endobronchial tumor. × 9.
FIG. 3. Chest X-ray of dog 22 mo after preparation showing trapped air in left lower lobe.

ily be checked after inserting the needle by drawing back the syringe plunger and checking for appearance of air bubbles. If the mucosa was punctured, the injection was made at a different site. The technic requires some practice but is usually mastered after a few attempts. The emulsion hardens quickly and produces a mass which projects into the bronchial lumen. Ten dogs were prepared as described above. Nine survived the procedure and remained in good health. The one fatality showed, at autopsy, an inadvertent penetration of the bronchial mucosa with deposition of the emulsion within the bronchial lumen and resultant extensive pneumonia. The remaining dogs were utilized frequently to demonstrate the lesions by bronchographic procedures. From 3 to 8 months after the preparations were made, 6 of the dogs were sacrificed periodically so that bronchial level of injection and structure of the lesions could be examined.

Results. Grossly, the endobronchial lesions were similar in appearance and resembled bronchial adenomata (Fig. 1). These pseudo-tumors varied in size but in general, degree of bronchial obstruction was proportional not only to amount of plastic injected but also to elapsed time after injection.

Histologically, the tumor (Fig. 2) was composed primarily of macrophages which contained ingested plastic. The macrophages tended to be arranged in groups separated by thin walled blood vessels and fibrous tissue. In older lesions, a thickened capsule and trabeculae of fibrous connective tissue became more prominent.

About 8 months after thoracotomy the 3 surviving dogs began to show clinical signs of bronchial obstruction as evidenced by periodic bouts of "asthma" (relieved by bronchodilators), enlargement of PA diameters with expiratory fixation of the involved side of the chest, and expiratory phase prolongation. Through a laboratory error, 2 of these dogs were lost to the study approximately 10 months after preparation and autopsies were not performed. One animal is still alive and shows a further progression of these symptoms. An X-ray taken 22 months after preparation of this dog's left lower lobe bronchus shows enlargement of the left chest, widening of the rib interspaces and scant lung markings resembling emphysema (Fig. 3).

Discussion. A method of producing endobronchial tumors has been described, and there is some evidence that these lesions produce obstructive emphysema. Eiseman, Petty and Silen(2) recently described a method of producing experimental emphysema and in addition carefully reviewed the historical background of this problem. Their criticisms of previous attempts to reproduce this disease in the experimental animal are valid, especially the commentary that "acute experiments are unlikely to produce the disease." We have felt for some time that successful production of experimental emphysema is probably dependent on production of a slowly developing, chronic, partial airway obstruction functioning over a prolonged period of time. Haidak and Curd(3) have suggested that injury to a bronchus due to trauma may result in chronic obstruction sufficient to produce expiratory bronchial collapse and thereby pulmonary emphysema. The method of Eiseman *et al.*(2) of inserting a small tube into the trachea to hinder expiration has limitations in that total lung tissue is involved. It is suggested that the slowly growing endobronchial lesion reported here represents a

physiological refinement over their method in that air can be trapped in localized lung areas while adjacent lung tissues will remain relatively collapsed and resilient as is known to occur clinically. In addition, these animals remain in good health for long periods of time and are available for various types of experiments.

Summary. Partially obstructive endobronchial lesions have been produced in dogs by transbronchial injection of a plastic material. These lesions were composed primarily of large accumulations of vacuolated macrophages. The size of these masses was apparently dependent upon their age. The implication of this type of lesion in possible production of experimental emphysema has been discussed.

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Corticosterone Levels in Adrenal Effluent Blood of Some Gallinaceous Birds.* (26011)

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Collection of adrenal effluent blood from mammalian species by cannulation of the adrenal vein(1) is facilitated by the morphological relation of this vessel to the vena cava. Recently Phillips and Jones(2) made direct collections of adrenal venous blood from the capon by a procedure that involved obstruction of the blood flow through the vena cava and formation of a pocket within this vein into which flowed adrenal effluent blood. This method, while satisfactory for some types of experimentation, is laborious for routine collection, is unsuitable for repetitive sampling, and is acutely traumatic.

In domestic fowl the adrenal veins are located on the ventral surface of the glands; the left adrenal vein passes transversely from the adrenal to the vena cava whereas the right adrenal vein passes dorso-laterally to the caval vein(3). The nature of the venous drainage from the *left gland* permits collection of adrenal effluent blood from the bird. However, the intimate apposition of the ovary to the left adrenal gland restricts sampling from this vein to the *male* galliform. We routinely make direct collections of adrenal venous blood by a simple, expedient manipulation that allows the animal to be used for study at a subsequent period.

Materials and methods. The male gallinaceous birds used in this study were 3 breeds of domestic fowl which were 10 to 20 weeks old and 1.5 to 2.5 kg in body weight; 5 to 12 month old ring-necked pheasants† about 1 kg in weight; and broad-breasted bronze turkeys† about 6 months old and approximately 12 to 14 kg in weight.

The birds were anesthetized by intravenous infusion of Nembutal. Peripheral blood was withdrawn either from the cephalic vein or from the heart. The body cavity was entered through incisions made in the skin and intercostal musculature between 6th and 7th ribs. A rib spreader was inserted and expanded to allow better access to the region of the adrenal gland. The presence of recrudesced gonads often necessitated retraction of the left testis for exposure of the adrenal vein. If sexually mature males are to be studied, it would be helpful unilaterally to castrate the animals.

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† Made available through the courtesy of Wisconsin Conservation Dept. and Univ. of Wisconsin Dept. of Poultry Husbandry, respectively.

TABLE I. Corticosterone Levels of Peripheral and Adrenal Effluent Plasma of Gallinaceous Birds.

Animal	No.	μg/100 ml plasma Adr. effl.	Peripheral
Chickens			
Wisco White	8	44.3 ± 5.5*	7.3 ± .3*
New Hampshire	8	48.8 ± 3.7	12.3 ±2.3
White Rock	8	37.0 ± 6.5	9.5 ± .2
Pheasants	19	34.8 ± 4.7	8.6 ± .8
" castrate	5	33.1 ± 7.2	7.8 ± .7
Turkeys	6	42.4 ± 5.3	7.8 ± .4

* Mean ± S.E.

Approximately 1.5 ml of blood were withdrawn in about 90 seconds from the left adrenal vein with a heparinized syringe fitted with a hypodermic needle the distal 5 mm of which had been bent to approximate a right angle. A 26 gauge needle was used for collecting adrenal effluent blood from the pheasant and domestic fowl, and a 22 gauge needle was used for the turkey. A pair of spectacle loupes is helpful for inserting the needle into the vein. Several samples can be obtained from the same bird if injury to the adrenal vein is kept at a minimum. Upon removal of the sample, the skin incision was closed with wound clips; the musculature between the costa was not sutured. The entire procedure can be completed in a matter of minutes.

In some instances blood was obtained from the turkey by cannulation. A 22 gauge hypodermic needle was separated from its shank, then inserted into one end of a piece of polyethylene tubing (0.034 inch I.D.). The cannula and bird were heparinized, and the needle was placed into the adrenal vein; blood was collected for 10 minute intervals.

The level of free corticosterone in aliquots of peripheral (1.0-2.0 ml) and adrenal effluent (0.2-0.4 ml) plasma was determined by the method of Silber *et al.*(4). Fluorescense was read in a Photovolt Corp. Fluorometer Model 54 and was quantitated relative to crystalline corticosterone. This method measures both corticosterone and hydrocortisone; however, we believe that the fluorescense attributable to the latter is negligible, since, as will be reported later, we were unable chromatographically to discern hydrocortisone in extracts of up to 50 ml of adrenal effluent plasma.

Results and discussion. Level of corticosterone in the sample of plasma is directly influenced by rate of removal of blood from the adrenal vein (Table II). Rapid withdrawal merely dilutes the level of corticosterone. However, if blood is withdrawn with a syringe at a rate of not more than 1.0 ml/min the amount of corticosterone present is not appreciably influenced by rate of flow of blood. Collection of blood with a syringe is more satisfactory for routine sampling than cannulation because of the ease by which blood can be obtained.

Plasma levels of corticosterone in the species of birds studied were surprisingly similar; values in adrenal effluent plasma were 4 to 6 times greater than peripheral levels (Table I). The amount of corticosterone in the peripheral plasma of the domestic fowl was similar to that reported by Urist and Deutsch (5). However, we found the level of corticosterone for adrenal blood of domestic fowl considerably less than the value Phillips and Jones(2) found in the capon. This difference may be due to variations in technics used for collecting blood and for determining amount of corticosterone. It is interesting to note that castration had no influence on adrenal activity in the pheasant (Table I).

Intravenous administration of 8 I.U. of ACTH (G. A. Breon & Co., El-Acorto Gel) to the pheasant increased the corticosterone in adrenal venous plasma 186% at 30 minutes and 253% at 60 minutes after injection (Table III). Wisco White cockerels showed in-

TABLE II. Effect of Rate of Blood Withdrawal on Plasma Concentration of Corticosterone in Turkeys.

Bird	Rate of blood removal (cc/min.)	Corticosterone (μg/100 ml)
I	.8	31.0
"	6.0	4.5
II	1.1	36.0
"	2.7	7.7
III	.2	28.8*
IV	.2	29.9*
V	.2	31.1*
"	.3	43.2*
"	.4	16.4*
"	.4	28.0*

* Sample obtained by cannulation of adrenal vein.

TABLE III. Corticosterone Levels in Adrenal Effluent after Repeated Bleedings and ACTH.

Animal		μg corticosterone/100 ml plasma at 0 time	30 min.	60 min.	90 min.
Pheasants					
No treatment	(3)*	25.7 ± 5.5†	34.0 ± 12.3†	31.0 ± 3.7†	
8 IU ACTH IV	(4)	29.6 ± 5.4	84.6 ± 34.5	104.6 (2)	
Chickens					
No treatment	(3)	27.9 ± 7.2	56.8 ± 15.3		26.3 ± 12.0
8 IU ACTH IV	(3)	30.7 ± 3.6	68.3 ± 14.5	143.7 ± 50.7	

* No. of animals. † Mean ± S.D.

creases of 123% and 368% at these same times. Urist and Deutsch(5), using the method of Silber, report an 85% increase in corticosterone level of peripheral plasma of male fowl following administration of 400 I.U. of ACTH. Phillips and Jones(2) found a decrease in amount of corticosterone in adrenal effluent blood of the capon following ACTH treatment.

Summary. A method is described for obtaining samples of adrenal venous blood from the bird. This allows avian species to be used for routine *in vivo* studies designed to assess adrenal activity by direct measurement. Levels of corticosterone, expressed as μg %, in adrenal effluent and peripheral plasma were 42.4 and 7.8 in turkeys and 34.8 and 8.6 in pheasants. In domestic fowl the values were 44.3 and 7.3 in Wisco Whites; 48.8 and 12.3 in New Hampshires, and 37.0 and 9.5 in White Rocks. ACTH (8 I.U. intravenously) increased the output of corticosterone from 29.6 to 104.6 μg % in pheasants and from 30.7 to 143.7 μg % in chickens 60 minutes after injection.

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Comparative Bilirubin Levels in Vitreous Body, Synovial Fluid, Cerebrospinal Fluid and Serum after Death. (26012)

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Further studies on postmortem chemistry (7-11) revealed in cases of jaundice presence of bilirubin not only in cerebrospinal fluid, where it was known to occur(1-6), but also in vitreous body and synovial fluid. This to our knowledge has not been reported before, and it seemed of interest, therefore, to obtain data on comparative concentrations in these fluids.

Materials and methods. Material from autopsies about 5½ hours after death on the average was based on a total of 78 male adult bodies including 44 cases of jaundice and 34 non-jaundice control cases. Spinal fluid was obtained by cisternal puncture(7,9,10), vitreous body by puncture of the eye balls at the outer canthus with a 20 gauge needle and 10 cc syringe(11), and synovial fluid was aspirated from the knee joints with a 16 gauge needle and 10 cc syringe. Bilirubin determinations were performed by the method of Malloy and Evelyn(13) with the usual 1 minute modification for direct bilirubin(13). Floccules of mucoprotein forming in dilute syno-

TABLE I. Comparative Averages and Ranges of Bilirubin Levels in Postmortem Vitreous Body, Cerebrospinal Fluid, Synovial Fluid and Serum.

	Values in jaundice			Postmortem normals			Antemortem normals	
		Bilirubin			Bilirubin		Bilirubin	
Body fluids	No. of cases	Total mg/100 ml	Direct mg/100 ml	No. of cases	Total mg/100 ml	Direct mg/100 ml	Total mg/100 ml	Direct mg/100 ml
Vitreous body	43	.04 ± .02 0 – .48	.01 ± .02 0 – .29	29	0	0	——no data——	
Spinal fluid	43	.23 ± .07 0 – 4.00	.11 ± .06 0 – 2.47	34	0	0	0	0
Synovial fluid	33	2.02 ± .31 0 – 10.5	1.11 ± .55 0 – 5.50	12	.28 ± .07 .15 – .75	.1 ± .04 0 – .48	——no data——	
Serum	43	8.6 ± .27 1.51 – 36.0	4.8 ± .34 .19 – 24.3	12	.9 ± .16[5] .2 – 1.5	.4 ± .09[5] .1 – .8	.62 ± .25[13] .2 – .8[5]	.11 ± .05[13] .1 – .2[8]

± indicates stand. dev. of mean.

vial fluid on acidification with diazo or blank reagent were resuspended by short vigorous shaking. The resulting bubbles usually rose rapidly enough to allow colorimetry after one minute.

Results. Table I summarizes averages and ranges of bilirubin concentrations in vitreous body, spinal fluid, synovial fluid and serum found in jaundice with bilirubin levels of more than 1.5 mg/100 ml and in normal non-jaundice cases with bilirubin levels of 1.5 mg/100 ml or less. Averages of total bilirubin in the present series of jaundice cases were 8.6 mg/100 ml in serum, 2.02 in synovial fluid, 0.23 in spinal fluid and 0.04 in vitreous body. As seen from the ranges, highest values of total bilirubin encountered in jaundice were 36.0 mg/100 ml in serum, 10.5 in synovial fluid, 4.0 in spinal fluid and 0.48 in vitreous. In normal non-jaundice cases free from hepatic, hemolytic and joint diseases and hemorrhagic contaminations bilirubin was generally absent in spinal fluid and vitreous but present in synovial fluid averaging 0.28 mg/100 ml for total bilirubin. This compared to normal serum values of 0.9 mg postmortem and 0.62 mg/100 ml antemortem. No data on normal antemortem synovial bilirubin were found in the literature nor statements on the presumed absence of pigment in the vitreous.

Ratios of bilirubin averages in vitreous, spinal and synovial fluid to serum bilirubin averages of the present jaundice cases (Table I) are presented in Table II. Total bilirubin values in the vitreous were 220 times smaller, in spinal fluid 35 times and in synovial fluid 4 times smaller than in serum. The direct bilirubin in vitreous was 480 times smaller than in serum, while the other direct bilirubin ratios approached or matched those of total bilirubin. By applying these ratios when serum bilirubin is known one may obtain an idea of what level could be expected in any of the other fluids. For instance, assuming a total serum bilirubin of 20 mg/100 ml, the expected bilirubin level in the vitreous would be 20/220 or about 0.1 mg, in spinal fluid 20/35 or about 0.5 mg and in synovial fluid 20/4 or about 5 mg/100 ml. Again, if 0.05 mg/100 ml were considered the limit of detectable bilirubin such traces could be expected in synovial fluid, spinal fluid and vitreous when serum bilirubin levels were about 0.05 x 4 = 0.2 mg, 0.05 x 35 = 1.8 mg and 0.05 x 220 = 11 mg/100 ml respectively.

It is obvious that the ratios given in Table II can not be more than approximations, and not infrequently exceptions from the rule will be found. In the present series traces of bilirubin have sometimes been observed in vitreous and spinal fluid when serum total bilirubin was less than 0.5 mg/100 ml. On the other hand, bilirubin was absent in vitreous and spinal fluid when total serum bilirubin

TABLE II. Ratios of Bilirubin Averages in Vitreous Body, Spinal and Synovial Fluid to Average Serum Bilirubin.

Bilirubin ratios of	Total bilirubin	Direct bilirubin
Vitreous body : Serum	1 : 220	1 : 480
Spinal fluid : Serum	1 : 35	1 : 45
Synovial fluid : Serum	1 : 4	1 : 4

was as high as 18.7 mg/100 ml. However, in general, bilirubin ratios will approach those given above.

Discussion. Occurrence of bilirubin in spinal fluid of the newborn(6), in jaundice (1-4,10) and after hemorrhages(2,4) has been known for some time. However, there seem to be no data in the literature about presence of bilirubin in the vitreous, although xanthochromia of the aqueous humor is mentioned by Krause(3). Yet there is evidence that its effect may have been noted clinically by observation of xanthopsia, yellow vision, which may occur in jaundice as well as santonin poisoning(12). The mechanism of xanthopsia in jaundice could be explained as an optic filter effect due to yellow coloration of the vitreous body and probably the other visual media.

No reports on bilirubin in synovial fluid were encountered except for contribution on use of bilirubin determinations for diagnosing a traumatic origin of joint effusions(14). In this connection it may be of interest that our material also included 4 cases in which synovial bilirubin was higher than serum bilirubin suggesting intraarticular production of part of the bilirubin from hemoglobin. In contrast to vitreous and spinal fluid there were high values of synovial bilirubin in jaundice, and non-jaundice cases showed it with sufficient frequency to conclude that bilirubin is a normal constituent of synovial fluid.

Ratios between total bilirubin of the fluids given in Table II indicate a diffusion gradient from vitreous to spinal fluid to synovial fluid. This effect may be due partly to the difference in surface of the diffusion membrane, which is smallest in the globular periphery of vitreous, much larger in choroid plexus and probably considerable in synovial villi.

Regarding the validity of postmortem chemistry for *intra vitam* application, bilirubin besides calcium has been found to be one of the stable substances after death(8,9). Since the ranges of normal postmortem and antemortem total and direct serum bilirubin are close, it may safely be assumed that the given postmortem bilirubin values in vitreous and synovial fluid will also approach closely those during life.

Summary. A total of 78 autopsies including 44 jaundice and 34 non-jaundice control cases have been studied for comparative bilirubin levels in vitreous body, spinal fluid, synovial fluid and serum. Ratios of total bilirubin averages in vitreous, spinal and synovial fluid to average total serum bilirubin were 1:220, 1:35 and 1:4 respectively. Highest and average values of total bilirubin found were 0.48 and 0.04 mg/100 ml in vitreous body and 10.5 and 2.02 mg/100 ml in synovial fluid respectively. Total synovial bilirubin in non-jaundice cases averaged 0.28 mg/100 ml. Bilirubin may thus be considered a normal constituent of synovial fluid.

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Use of Optical Density of Fluorescent Conjugates for Analysis of Co-Precipitating Antibody.* (26013)

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The possibility that concentration of fluorescein in fluorescein-conjugated protein solutions might be used as an index of their protein content was suggested by the work of Schiller *et al.*(1), who demonstrated that fluorescein-protein conjugates, like fluorescein dye, have a light absorption peak at a wave length around 490 mμ. Optical density (O.D.) at 490 mμ of a fluorescein-protein conjugate is an index of its dye content and, for that conjugate, of its protein concentration. For any preparation this direct relationship exists over a wide enough range to suggest the spectrophotometric determination of fluorescein content as a means of determining protein content.

A procedure for quantitative determination of non-precipitating antibodies has been described(2). Total protein precipitated in the equivalence zone from a mixture of precipitating and non-precipitating antibodies and homologous antigen is compared to amount of precipitate obtained by using precipitating antibody and antigen alone. The difference is taken as the amount of non-precipitating antibody [(precipitins + co-precipitating antibodies + antigen)—(precipitins + antigen) equals co-precipitating antibodies]. The validity of the results depends on the assumption that addition of the co-precipitating factor does not alter amount of protein precipitated from the precipitating serum.

Since concentration of the fluorescein-protein conjugate can be estimated directly on the basis of the O.D. at 490 mμ, use of conjugated antibodies (precipitins in the system described above) should obviate the need of the double sample. We are reporting preliminary experiments for titrating conjugated added to unconjugated precipitating antibodies.

Procedure. Anti-egg albumin and anti-human globulin rabbit antisera were obtained from animals inoculated weekly with 5 subcutaneous injections of antigen† in Freund's complete adjuvant. One portion of each serum was precipitated at 33% saturation with ammonium sulphate to obtain the crude gammaglobulins that were conjugated with fluorescein isothiocyanate following the method of Riggs *et al.*(3).

After 7 days' dialysis against phosphate buffered saline at pH 7.4, variable amounts of the conjugates were dissolved in N/1 NaOH and the solutions were examined for the spectrum absorption between 300 and 1000 mμ with a Beckman DU Spectrophotometer, against a blank of N/1 NaOH. Fig. 1 shows that in this alkaline medium conjugates have a sharp absorption peak between 420 and 540 mμ with a maximum near 500 mμ.

The absorption characteristics of the various concentrations of the fluorescent protein preparations were determined by their O.D. at 490 mμ. Results were proportional to their protein concentration for a range of 5-200 γ/ml.

Because the antibodies, specifically precipitated from the conjugated gamma globulins and then redissolved in N/1 NaOH, had the same fluorescein-protein ratio as total conjugated gamma globulins from the same batch the standard was always prepared with crude conjugated gamma globulins.

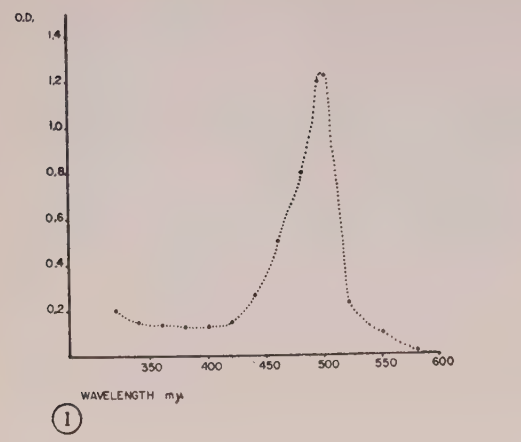
Protein content of the standard was determined by the Lowry method(4) at 750 mμ and the O.D. of known dilutions of the same preparation, determined at 490 mμ was plotted against their protein content (Fig. 2).

The following antigen/antibody systems were prepared:

a) unconjugated rabbit anti-egg albumin serum + conjugated rabbit anti-egg albumin globulins + egg albumin.

* This work was supported by U. S. Public Health Service grant.

† Thrice crystallized egg albumin (Armour) poliomyelitis immune human globulin (Lederle).



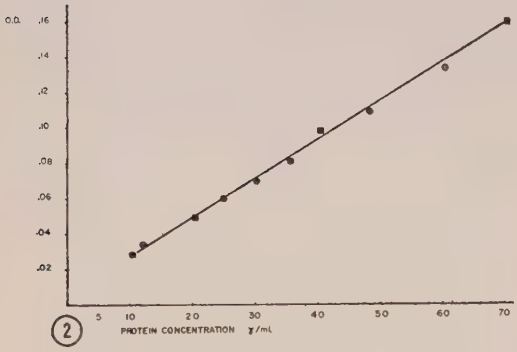


FIG. 1. Absorption spectrum of a fluorescein isothiocyanate conjugated rabbit anti-human globulin solution in N/1 NaOH. Concentration of protein 200 γ/ml.

FIG. 2. Relation between optical density and protein concentration in a conjugated anti-egg albumin (○) and in a conjugated anti-human globulins (■) rabbit globulin solutions.

b) unconjugated rabbit anti-egg albumin serum + conjugated anti-human rabbit globulins + egg albumin.

c) unconjugated rabbit anti-human serum + conjugated rabbit antihuman globulins + human globulins.

d) unconjugated rabbit anti-human serum + conjugated rabbit anti-egg globulins + human globulins.

The conjugated globulins were in the concentration of 1% protein, the heterologous conjugated antibody in b) and in d) served as controls.

The various mixtures were placed at 37°C for 2 hr, then overnight at 4°C. Resulting precipitates were washed 3 times with saline buffered at pH 7.4 at 4°C, then dissolved in N/1 NaOH.

One part of the dissolved precipitate was used for spectrophotometric determination of the conjugated protein content by means of the O.D. at 490 mμ using the standard curve of O.D. *vs.* protein concentration prepared from the same batch. The other part of the dissolved precipitate was used to estimate protein content by the Lowry method. Volumes used for the latter were calculated on the basis of the expected amount of specific precipitate so that the values were in the workable range of the Lowry method either at 750 or 560 mμ. The 490 mμ more suitable for higher protein concentrations was not used because of interference of the fluorescent dye.

Results. The results of a typical determination are shown in Table I. Only when homologous conjugated antibody was added did an appreciable amount of fluorescent dye appear in the dissolved precipitate. No significant O.D. readings could be obtained at 490 mμ in the redissolved precipitate from the unconjugated antibody to which heterologous conjugated antibody and specific antigen had been added.

Amount of protein precipitated from the conjugated globulins was calculated by 2 methods: (1) using the Lowry technic and subtracting the results of b) from a) and of d) from c) respectively; (2) by direct estimation of O.D. of the precipitate at 490 mμ, interpolating this result on the standard. It should be emphasized that the results obtained by the two methods agree only when the data in the equivalence zone are compared; at other antigen/antibody ratios the data, calculated by difference, do not agree with the direct spectrophotometric determination. In Table I the results obtained in equivalence zone are italicized.

The experiment has been repeated using unconjugated rabbit antiserum heated at 75°C to destroy its precipitating activity without eliminating its capacity of co-precipitating in presence of homologous precipitating antibody and antigen(5). Table II shows that, in this condition too, use of fluorescein conjugated precipitins permits us to distinguish in

TABLE I. Analysis of Specific Precipitate Obtained from Mixtures of Unconjugated Precipitins and Conjugated Precipitins.

Antibodies	Antigen (γ)	Total pptd. proteins (ag + ab) (γ)	O.D. at 490 mμ	Antibodies pptd. (γ)	Conjugated proteins in precipitate (γ)	Unconjugated proteins in precipitate (γ)
.1 ml anti-egg serum	200 EA	600	.045		72	
+ .3 ml conjug.	150	820	.061		104	
anti-egg glob.	100	*960*	.080	*860*	*134*	*726*
(1% proteins)	75	860	.075	785	120	665
	50	800	.065	750	110	640
.1 ml anti-egg serum	200 EA	400	.005		traces	
+ .3 ml conjug.	150	650	—		—	
anti-hum. glob.	100	820	—	(720)	—	
(1% proteins)	75	790	.005	(715)	traces	
	50	*780*	—	*730*	—	
.1 ml anti-hum. serum	100 HG	390	.078		128	
+ .3 ml conjug.	75	*510*	.096	*435*	*152*	*283*
anti-hum. glob.	50	420	.074	370	124	246
(1% proteins)	25	370	.071	345	116	229
.1 ml anti-hum. serum	100 HG	230	—		—	
+ .3 ml conjug.	75	260	.002	(185)	traces	
anti-egg glob.	50	*340*	.006	*290*	"	
(1% proteins)	25	250	.005	225	"	

Washed precipitates were dissolved in 4 ml of N/1 NaOH; the Lowry method was performed on .2 ml of these solutions and results multiplied by 20; O.D. were interpolated on standard curve to obtain conjugated protein concentration and results multiplied by 4. Proteins precipitated from unconjugated serum were given by the difference: Antibodies pptd. – conjugated proteins pptd.

EA = egg albumin; HG = human globulins.

TABLE II. Analysis of Specific Precipitate Obtained from Mixtures of Conjugated Precipitins and Heated Rabbit Antisera.

Antibodies	Antigen (γ)	Total pptd. proteins (ag + ab) (γ)	O.D. at 490 mμ	Antibodies pptd. (γ)	Conjugated proteins in precipitate (γ)	Unconjugated proteins in precipitate (γ)
.5 ml anti-human	100 HG	310	.071		130	
heated serum +	75	*340*	.093	*265*	*148*	*117*
.3 ml conjugated	50	320	.070	270	114	156
anti-human glob.	25	210	.066	185	108	77
(1% proteins)						
.5 ml anti-egg heated	100 HG	160	.075		120	
serum + .3 ml	75	190	.069		114	
conjugated anti-	50	220	.090	(170)	144	(16)
human glob.	25	*190*	.089	*165*	*142*	*23*
(1% proteins)						

Anti-human globulins and anti-egg albumin rabbit sera, diluted 1 to 5, were heated at 75°C for 15′, kept in cold for 2 wk and centrifuged before using; no precipitins were present in these sera. The mixtures unconjugated heated antisera, conjugated anti-human globulins, human globulins were kept for 3 days at 4°C before titration of precipitates.

the specific precipitate the proteins of the two sera.

The optical characteristics of the fluorescein isothiocyanate might be applicable for determination of protein content of non-precipitating antibodies when they are co-precipitated by fluorescein-conjugated precipitins. We are at present trying to apply this method to quantitative determination of the reagins.

Summary. The O.D. of fluorescein isothiocyanate permits determination of quantity of protein derived from a conjugated precipitin serum when this is added to an unconjugated precipitin serum. This permits one to distinguish in a specific precipitate the antibodies of

the 2 sera. This finding could have practical application in co-precipitation technics.

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Experimental Histoplasmosis in Large Domestic Animals.* (26014)

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Previous studies from these laboratories have been concerned with pathogenesis of histoplasmosis in mice(1-4), dogs(5,6), and monkeys(7). The present report describes experimental disease in horses, cattle, sheep, and swine. Although naturally occurring clinical histoplasmosis is rare in these species(8), skin-test and other evidence(9) suggest that, as in man, asymptomatic infection may be more common. To our knowledge, experimental infection of these species with *H. capsulatum* has not been reported.

Materials and methods. Eight healthy young adult animals, 2 of each species and one of each sex, were used. After completion of base line studies, each was inoculated intratracheally(10) with 5.0 ml of saline suspension of yeast phase *H. capsulatum*† containing 2.0 ml of packed cells from 3-day cultures grown on brain heart infusion (Difco) blood agar. Pontocaine (Winthrop), 2.0 ml, was administered intratracheally 5 minutes prior to inoculation of yeast cells to depress reflex coughing. Animals were observed daily for clinical signs of illness, and blood samples for cultures, complete blood counts, and serologic studies were taken 7, 10, 17 and 24 days after inoculation. Serologic response was studied by collodion agglutination(11) and complement fixation(12) tests at these intervals, then weekly for about 3 months. Skin tests were performed 31 and 73 days after inoculation, using histoplasmin prepared and standardized in infected dogs by previously described methods(13-14). Seven of 8 animals were sacrificed about 3 months post-inoculation, and complete autopsies performed. Tissues taken at autopsy and all blood samples were treated and cultured by the method of Conant(15).

Results. Table I summarizes findings in 8 large domestic animals inoculated intratracheally with *H. capsulatum.* All except the female sheep exhibited clinical evidence of infection and all produced antibodies and developed positive skin tests as compared to pre-inoculation antibody-negative sera and negative skin tests. Fever was first observed in 7 of 8 animals 3-5 days after inoculation, and persisted for about 1 week except in the male sheep and swine, in which 3 weeks of fever was observed. Symptoms varied from slight cough in the 2 swine to definite respiratory symptoms in the male sheep characterized by frequent cough, dyspnea, dry rales, tachypnea and hyperpnea; rales and hyperpnea persisted in this animal up to time of sacrifice at 88 days. A positive blood culture was obtained from the male sheep 7 days after inoculation, and *H. capsulatum* was seen in lung lesions at autopsy. In other animals, symptoms subsided within a few days after the end of the febrile period. A positive blood culture was also obtained from the female swine 7 days after inoculation. No significant

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† Strain G 13—an isolate from spontaneous histoplasmosis in a dog.

TABLE I. Response of Large Domestic Animals to Viable Yeast Phase *H. capsulatum* Given Intratracheally.

Animal	Sex	Signs and symptoms*	Blood culture P.I.D.† pos.	Reciprocal of antibody titer HCA‡	Reciprocal of antibody titer YPCF‡	Skin test P.I.D. pos.	Disposition		Histoplasma seen in tissue
Horse	♀	FC	—	80	160	31-73	Died	110§	+
"	♂	FCDR	—	320	1280	31-73	Sacrificed	87	—
Cattle	♀	FCRT	—	10	160	31-73	"	91	—
"	♂	FCDR	—	10	320	31-73	"	92	—
Sheep	♀	None	—	40	320	31-73	"	88	—
"	♂	FCDRTH	7	80	1280	31-73	"	88	+
Swine	♀	FC	7	10	80	31-75	"	88	—
"	♂	FC	—	10	80	31-73	"	88	—

* F, C, D, R, T, H = Fever, cough, dyspnea, râles, tachypnea, hyperpnea.
† P.I.D. = Post-inoculation day.
‡ HCA = Histoplasmin collodion agglutination; YPCF = Yeast-phase complement fixation; pre-inoculation titers all negative.
§ Rechallenged I.T. on 75th P.I.D. Rechallenged I.V. on 98th P.I.D.

hematologic changes were seen in any of the animals during observation period.

The female horse was rechallenged, intratracheally, 75 days after first inoculation. No clinical signs of infection were seen after rechallenge and no increase in antibody titer occurred. The animal was challenged again, intravenously, with the same dose used intratracheally, on 98th day. After 3 days it became depressed, markedly febrile, hyperpneic, anorectic, and began to lose weight. Death occurred 12 days after intravenous challenge. Histopathologic studies revealed presence of diffuse granulomatous pneumonia superimposed upon foci with calcified centers and extensive peripheral fibrosis of more than 12 days standing. Bodies identified as *H. capsulatum* were seen in giant cells in both types of lesion. The tubercle-like foci appeared to be the result of earlier intratracheal inoculation, whereas the diffuse, active pneumonia was apparently produced by the intravenous challenge.

Gross and histopathologic findings compatible with histoplasmosis were obtained also in both sheep and in the female swine. The male sheep also had multiple focal granulomatous orchitis, with lesions similar to those produced in the lungs and seen in other species with histoplasmosis. Microscopic pathology was less specific in both cattle, the male horse, and the male swine. *H. capsulatum* was not cultured from any of the animals at autopsy and in only 2 cases (Table I) were organisms seen in tissue sections.

Discussion. The above results indicate that horses, cattle, sheep and swine are susceptible to *H. capsulatum* given intratracheally. However, clinical recovery of the animals, paucity of viable organisms in post-mortem tissues, and localization of lesions by fibrosis point to natural ability to cope with the infective agent. In contrast, dogs similarly inoculated in these laboratories(5) developed acute disseminated, fatal histoplasmosis and the organism was readily isolated from the blood and from tissues taken at autopsy. Intravenous challenge in a horse resulted in death 12 days later. This route of inoculation also produced rapidly fatal infection in monkeys (7).

These experiments provide proof that large domestic animals insensitive to histoplasmin can convert to skin-test positivity after exposure to *H. capsulatum,* a finding which supports results observed in the survey of farm animals with histoplasmin. Menges(9) skin-tested horses, cattle, sheep and swine in Missouri and found 73%, 12%, 34% and 2%, respectively, to be reactors.

Summary. Intratracheal inoculation of yeast phase *H. capsulatum* produced non-fatal infection in horses, cattle, sheep and swine as determined by clinical response, development of positive skin tests, production of antibodies, or histopathologic changes. Intrave-

nous inoculation caused fatal infection in a horse.

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Mucopolysaccharides of Aorta at Various Ages.* (26015)

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Changes in the patterns of mucopolysaccharides (MPS) of cartilage, nucleus pulposus, skin and aorta, concomitant with ageing, have been described in past years. In general, these studies indicated a decrease of total MPS per unit weight with chronological age coincident with an increase in collagen content. The most profound changes were encountered in human costal cartilage(1). In this tissue, a steady decrease in total MPS was observed. Keratosulfate increased in the first two decades and remained constant thereafter. Chondroitin sulfate steadily decreased with time and chondroitin-4 sulfate (ChS-A) was replaced by chondroitin-6 sulfate (ChS-C). Similar changes have been described in degenerating versus normal nucleus pulposus(2). The pattern of age changes in the MPS of skin has also been described.

While these studies were based on isolation and chemical and enzymatic characterization of the isolated fractions, the reported studies of ageing of aorta have been based on analysis of the whole organ for hexosamine, uronic acid, sulfate, and identification of monosaccharides by paper chromatography after acid hydrolysis, as for example in the work of Dyrbye and Kirk(3,4,5). Those authors found until age 60 no change in ratio of galactosamine to glucosamine, and after 60 a slight increase. Similarly, the sulfate content of the tissues was found not to change till age 60. More recently Kirk has reported a crude MPS fraction of aorta to have the anticoagulant activity of 1% standard heparin(3) while Yü and Blumenthal found approximately 4%(6). Jorpes(7) and others have obtained heparin in small quantities from aorta.

The MPS of bovine and human aortae have been isolated and characterized. In bovine abdominal aorta of 1- to 2-year-old cattle, total isolated MPS was approximately 1% of defatted dry weight. Of this total, ChS-A represented 45%, hyaluronic acid 23%, and ChS-B and heparitin sulfate approximately 16% each(8).

The present study was undertaken to com-

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pare MPS patterns of isolated fractions in whole human aortae at various ages.

Method. Thirty-three fresh human aortae were obtained at autopsy and segregated into 7 different age groups, containing from 3 to 7 aortae in each group, with average ages of the groups ranging from 21 to 72 years. The tissues showed a general trend of an increasing degree of atherosclerosis with increasing age. The groups of average age 46 and 67 were all female, the 21-year-old group was of mixed sex, and the other 4 groups were male. The aortae in each group were dried in acetone, cleaned of perivascular and adventitial tissue, cut in small pieces, ground in a mill and treated further with acetone. The powders of each aorta were weighed and combined so that each group contained approximately 35 g of dry aorta. This was homogenized (VirTis) in 0.1 N HCl, digested with pepsin, neutralized and digested with trypsin(8), and purified by means of the Sevag procedure, absorption with Lloyd's reagent/kaolin mixture and several alcohol precipitations(8). It was finally fractionated as the calcium salt at 18%, 23%, 28%, 33%, 43%, and 52% alcohol.

The fractions obtained above were analyzed for uronic acid by both the carbazol and orcinol methods(9), for total hexosamine by the method of Elson and Morgan(9), and for glucosamine and galactosamine by Fischer-Dorfel paper chromatography(10). In addition, increase in reducing sugar was measured after enzymatic digestion with combinations of pneumococcal hyaluronidase, testicular hyaluronidase, and A-adapted (this extract digests ChS-B when the organism has been adapted to either ChS-A or B(11)) and heparitin-adapted enzyme. Rarely did any one fraction contain only one polysaccharide, and further separation was required to obtain a quantitative estimation of amount of each MPS.

The 18% and 23% fractions were each found to contain a mixture of ChS-B and heparitin sulfate. They were therefore combined and separated by the method of Cifonelli and Dorfman(12). The 28% fraction was usually small and not susceptible to further separatory procedures. It was therefore necessary to rely on a correlation of the results of the paper chromatogram and enzyme experiments. The 33% fraction contained hyaluronate and estimation of the amount with pneumococcal hyaluronidase correlated well with the actual amount isolated by the method of Scott(13). The residual MPS was identified by infrared spectrophotometry. The 43% fraction usually contained only ChS and was identified again by its infrared absorption. There was no significant precipitation above 43%.

From these data, relative amounts of the different MPS were calculated.

Results. Total yield of MPS showed no correlation with age (nor did total hexosamine done on individual aortas) and varied between 0.85% and 1.2% of the dry weight of aorta. In the following graphs the data are expressed as per cent of each polysaccharide of total MPS against age in years (Fig. 1-4). It can be seen that ChS-B and heparitin sulfate increase with increasing age (and increasing degree of atherosclerosis). Essentially the reverse is the case for hyaluronate and ChS-C.

Of interest in regard to comparison with the data of others cited above is that one can deduce relative amounts of glucosamine and galactosamine of the aortae for each age group—and this does indeed give a quite constant value. For the 7 age groups, in order of increasing age, ratio of galactosamine to glucosamine (ChS-C + ChS-B to HA + heparitin) would be 74/26, 69/31, 74/26, 74/26, 74/26, 70/30, 74/26.

Heparin, keratosulfate, and ChS-A could not be identified in any fraction.

Discussion. Isolation and fractionation of MPS of aorta present greater difficulties than that from any other tissue studied thus far. The reasons for these difficulties are not obvious and may reside, in part, in the protein complexes in which they occur in vascular tissues. By reason of the abnormal behavior towards alcohol fractionation, it is necessary to make the conditions of isolation and fractionation as uniform as possible. Even so, occasionally the 28% alcohol concentration appeared to precipitate all 4 fractions.

The results of this investigation show a pattern of ageing totally different from that encountered in costal cartilage. In the latter, chronological ageing is accompanied by

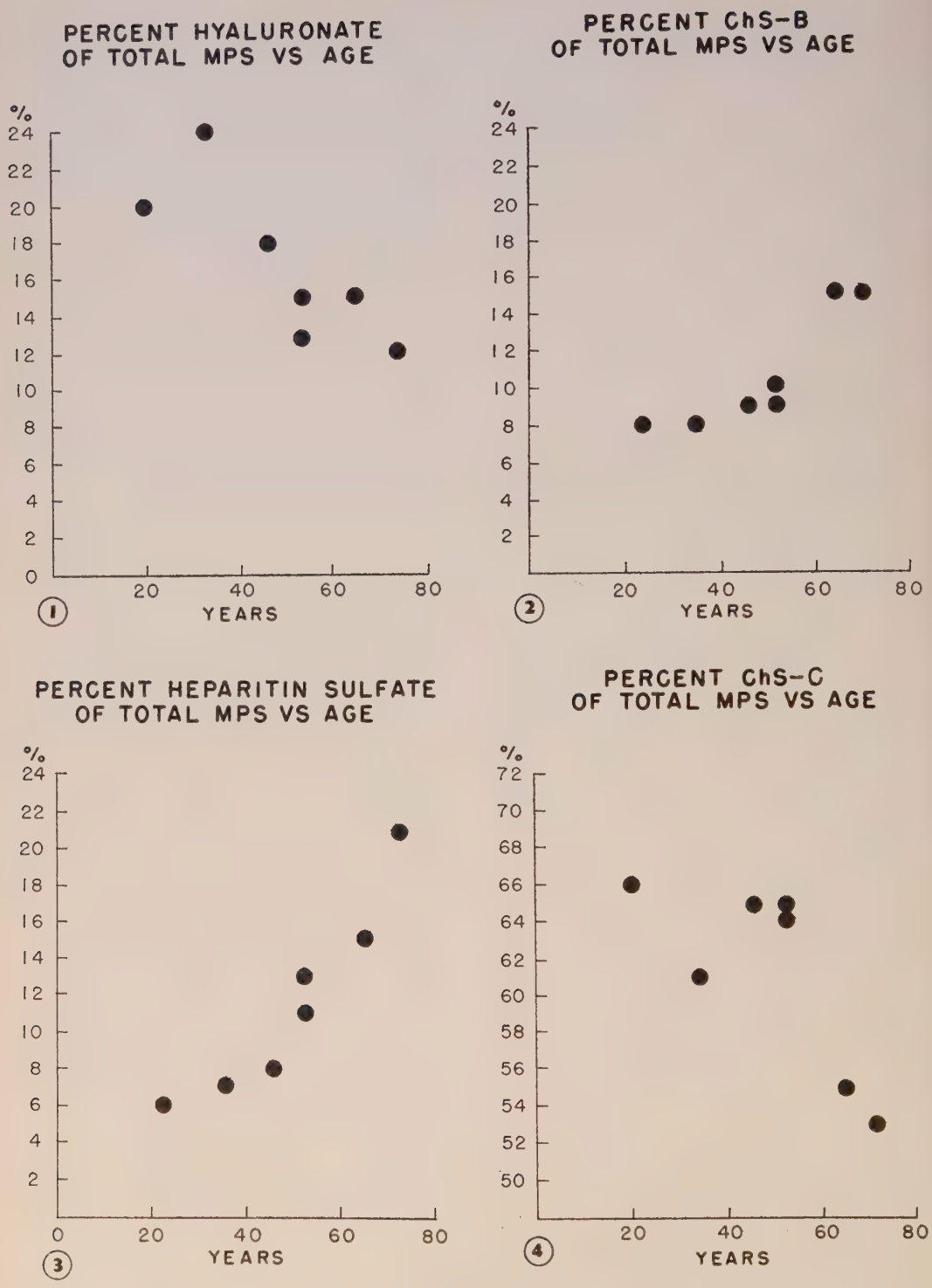
PERCENT HYALURONATE
OF TOTAL MPS VS AGE
%
24
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
YEARS
1
PERCENT ChS-B
OF TOTAL MPS VS AGE
%
24
22
20
18
16
14
12
10
8
6
4
2
20
40
60
80
YEARS
2
PERCENT HEPARITIN SULFATE
OF TOTAL MPS VS AGE
%
24
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
YEARS
3
PERCENT ChS-C
OF TOTAL MPS VS AGE
%
72
70
68
66
64
62
60
58
56
54
52
50
20
40
60
80
YEARS
4

FIG. 1-4.

profound qualitative changes in the types, besides a decrease in total MPS. Ageing of pig skin appears to be characterized by a decrease in hyaluronic acid content and an increase in chondroitin sulfate B. In aorta, while the histologically demonstrated changes in elastic and collagenous components are very striking, the changes in the MPS are more subtle and complex. The complexity can be assumed to be a result of ageing *per se*, of degenerative and reparative processes occurring simultaneously. The decrease in hyaluronate and ChS-C is similar to that found in other ageing tissues and perhaps would be still more marked if ageing without inflammatory and reparative processes could be studied. In other publications, the association of hyaluronate with elastic fibers was indicated(14). In fact, the known fragmentation of elastic fibers with ageing may well be a consequence of changes in this component. A study of aorta in species not subject to atherosclerosis probably would allow a clearer picture of the process in man.‡

Summary. Mucopolysaccharides were isolated from whole human aortae and characterized by analysis, enzymatic digestion, optical rotation and I.R. spectroscopy. Average age of the groups ranged from 21 to 72 years. Total MPS and galactosamine/glucosamine ratios showed no significant change with age. Of the 4 MPS fractions identified, hyaluronate and ChS-C decreased with age while ChS-B and heparitin sulfate increased. The data are discussed in relation to age changes in other tissues of mesodermal origin. It is assumed that the complex pattern of human aorta is the resultant of age changes *per se* superimposed on degenerative and reparative processes.

We wish to thank the Dept. of Pathology for their cooperation in obtaining aorta.

‡ After completion of this manuscript, a study of MPS of human aorta with and without arteriosclerosis was published by Buddecke(15). The author obtains a mucoid fraction (on phenol extraction) similar to serum mucoid. His findings regarding the acid MPS disagree substantially with our own. Thus, he finds no heparitin sulfate fraction, which has been isolated from bovine and all human aorta studied, but a substantial fraction which he designates as keratosulfate. Methods of isolation and characteristics of the fractions are not sufficiently documented to permit their identification.

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On the Control of Antheridium Formation in the Fern Species *Lygodium japonicum.** (26016)

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Döpp(1) demonstrated that an extract from mature prothalli of *Pteridium aquilinum* hastened the onset of antheridium formation in young prothalli of this fern species by a few days and in prothalli of *Athyrium filix-mas* by a few weeks. Döpp envisaged the possibility that the promotion of antheridium formation occurred by way of nonspecific growth inhibition. Subsequent investigations, though, led to the conclusion that the activity of the extract must be attributed to a specific factor which controls initiation of antheridia during the normal process of development(2,3).

An assay was devised which took advantage of the observation that prothalli of *Onoclea sensibilis* failed to form antheridia spontaneously under the prevailing conditions of culture but responded readily when grown on a medium containing extract of mature *Pteridium prothalli*(2). Conditions were further defined under which the extract from 7-week-old prothalli of *P. aquilinum* was usually active to a dilution of 1:30,000. This increased the activity obtained by Döpp about 300 times. Under these same conditions of culture the active substance accumulated to almost as high an activity in the medium(2). The active factor was recently obtained in a highly purified form by Pringle *et al.*(4). It was concluded from these studies that the inducing factor must be active at a concentration of less than 1 part in 10 billion (10^{-4} μg/ml).

Although prothalli of both *O. sensibilis* and *P. aquilinum* responded readily to the active factor at young stages of development, they lost their responsiveness soon after they attained heart shape and a few days prior to attaining the archegonial phase. Once insensitive, the prothalli failed to form antheridia even if supplied with the factor at a concentration 15,000 times higher than that sufficient to induce antheridia in prothalli just a few days younger(3).

Further investigations led to the conclusion that prothalli do not begin to produce the factor at effective concentrations until after they have become insensitive to it. Thus, the antheridia a prothallus forms arise in response not to antheridial factor produced by itself, but to antheridial factor elaborated and secreted into the medium by the more rapidly growing and developing individuals of the gametophyte population(3).

Studies on the activity spectrum of this substance (termed *Pteridium* factor) disclosed that *Pteridium* medium was active toward the tested representatives of 8 out of 9 subgroups of the family Polypodiaceae: Asplenioids, Pteroids(1); Onocleoids, Blechnoids, Dryopteroids(2); Gymnogrammoids (5), Woodsioids(6) and in *Nephrolepis hirsutula* (Davallioids). Among nonpolypodiaceous species only *Dennstaedtia punctilobula* (Dicksoniaceae) was responsive. The substance failed to promote antheridium formation even at the highest available concentration (½ full strength *Pteridium* medium that was active toward prothalli of *O. sensibilis* to a dilution of 1:30,000) in the following fern species: *Polypodium aureum* (Polypodiaceae), *Lygodium japonicum, Anemia phyllitidis, Mohria caffrorum* (Schizaeaceae), *Osmunda claytonia, O. cinnamomea* (Osmundaceae) and *Alsophila australis* (Cyatheaceae).

Subsequent investigations led to demonstration of a further substance (termed *Anemia* factor) which controls formation of antheridia in *A. phyllitidis,* one of the several species found to be unresponsive toward the *Pteridium* factor(6).

The present account is concerned with control of antheridium formation in *L. japonicum* which, like *A. phyllitidis,* belongs to the family Schizaeaceae.

Methods. The procedures used in sterilization and inoculation of the spores have been

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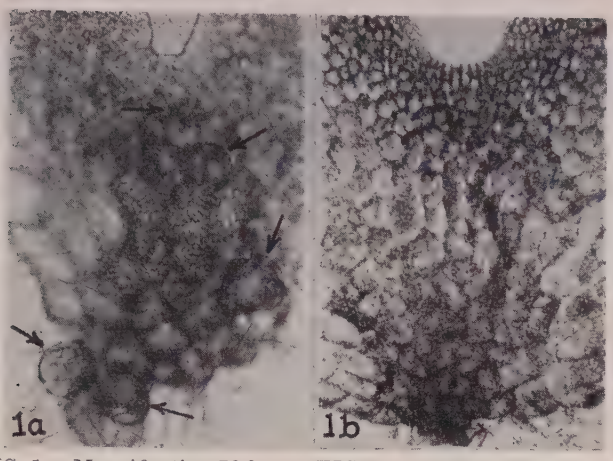

FIG. 1a. Magnification 76.8 ×. FIG. 1b. Magnification 33.6 ×.

described(3). This same report gives a detailed description of the conditions of culture and of the medium which contains the inorganic salts used by Moore(7). Hoagland's earlier-used A-Z solution of microelements was replaced with a Hoagland trace element solution of simpler composition (mg/l of medium: H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.22, MoO_3 0.07, $CuSO_4 \cdot 5H_2O$ 0.08). Iron was added as ferric tartrate. The prothalli were cultured in 125-ml flasks, each containing 33 ml of medium, or in 50-ml flasks with 12 ml of nutrient. Unless stated otherwise, the media were solidified with 1% agar.

Results. It was found that spores of *L. japonicum*, superinoculated on 25- or 40-day-old gametophyte cultures of this same species, gave rise to prothalli that attained the antheridial phase at a very early stage of development. Prothalli superinoculated on 15-day-old cultures attained the antheridial phase somewhat later although still several days prior to control prothalli.

This observation suggested that as the prothalli of *L. japonicum* reached a certain stage of development, they began to elaborate, and to secrete into the medium, a substance controlling induction of antheridia. This hypothesis received support when medium that had supported the growth of *Lygodium* prothalli was assayed for antheridium-inducing activity. The assays were conducted by inoculating spores of *L. japonicum* on fresh nutrient medium which contained medium of *Lygodium* cultures. The *Lygodium* medium was added at a dilution series with a dilution factor of approximately 3 (1/3, 1/10, 1/30, 1/100, . . .). Fifty-ml flasks which contained 12 ml of medium were used. Assays were read 15 days after inoculation, 6-7 days prior to onset of spontaneous antheridium formation. Medium from 15-day-old cultures or younger cultures was regularly inactive even at ⅓ full strength, the highest concentration of *Lygodium* medium tested. The medium of 25-day-old cultures, however, induced antheridium formation to a dilution of 1/10, the medium of 30-day-old cultures to a dilution of about 1/30, and media of 40- and 50-day-old cultures to a dilution of 1:100 (occasionally 1/300), and 1:300 (occasionally 1/1000), respectively. Fig. 1a shows a 14-day-old prothallus with 5 antheridia (see arrows) which were initiated in response to 1/30 full strength medium of a 40-day-old *Lygodium* culture, while control prothalli have not yet attained the antheridial phase even 19 days after inoculation (Fig. 1b). Whereas the lowest effective concentration of *Lygodium* medium brought about antheridium formation in only 1 out of 4 to 20 prothalli, the percentage of responding prothalli rose to 100 as concentration of the active factor was increased by a factor of from 3 to 10. Antheridial initials could be first discerned about 11 days after the spores were inoculated (the first spores germinated about 5 days following their inoculation), about 10 to 11 days prior to their appearance in control cultures.

Bauke(8) and Heim(9) reported that *L. japonicum,* unlike most other fern species, begins to form archegonia before it attains the antheridial phase. Archegonium-bearing prothalli first appeared in our cultures about 20 to 21 days following inoculation and 1 or 2 further days elapsed until gametophytes with antheridium initials could be seen. The prothalli which had just initiated the first antheridium all bore either one or, mostly, 2 archegonium initials. Thus, these prothalli actually attained the archegonial phase shortly before the first antheridium initial could be seen. Inspection of slightly older cultures, though, showed that the observations of Bauke and Heim were incomplete. All prothalli bore antheridia in about 25-day-old cultures but the smaller ones, *i.e.,* the most slowly growing individuals of the gametophyte population, lacked archegonia. Within a few further days, however, all prothalli bore sex organs of both types. It is inferred from this that the sequence of sex organ formation encountered in the prothalli that first attain the reproductive phase is reversed in more slowly growing individuals of the gametophyte population. This reversal may be understood if we consider that the most rapidly growing prothalli first attain the developmental stage at which elaboration of the antheridium-inducing factor, and its secretion into the medium, sets in. It may thus be postulated that the more slowly growing individuals begin to form antheridia at an earlier stage of development because they respond to this secreted factor before they themselves begin to produce it. This hypothesis was considered established with the demonstration, firstly, that onset of the antheridial phase preceded attainment of the archegonial phase in all prothalli if the medium was supplemented with the antheridium-inducing factor and, secondly, that the opposite sequence prevailed in all prothalli if they were picked randomly from young (13-day-old) cultures and isolated, one per flask, on new medium.

The zone of spontaneous antheridium formation in *L. japonicum* is, according to Bauke and Heim(8,9), restricted to the anterior part of the gametophyte. While this could be confirmed for the majority of gametophytes, a few of the very smallest individuals occasionally bore antheridia in the posterior region.

Moreover, if spores of *L. japonicum* were inoculated on 30-day-old cultures of this same fern species or on medium supplemented with antheridium-inducing factor, antheridia were observed to occur in the basal region, even among the very basal cells, of all gametophytes. As these prothalli continued to grow, the zone of newly formed antheridia became gradually restricted to the anterior portion of the gametophyte, indicating that the basal cells were sensitive to the antheridium-inducing factor during an initial period but lost their responsiveness as they matured. This hypothesis was considered established with the demonstration that antheridia arose on the basal parts of all gametophytes if antheridial factor (0.5 ml of ½ full strength medium from 40-day-old *Lygodium* cultures that was active to a dilution of 1:300) was added to 7-day-old cultures in 50-ml flasks, while occurrence of antheridia was restricted to the anterior region if the same amount of antheridium-inducing factor was added to 14-day-old cultures (again with the exception of the very smallest, *i.e.,* developmentally least advanced, individuals).

The *Lygodium* factor failed to promote antheridium formation in prothalli of *O. claytonia* (as checked 15 days after inoculation, 2 days prior to onset of spontaneous antheridium formation) which were also unresponsive to the *Pteridium* factor and the *Anemia* factor(6).

The antheridium-inducing activity of the *Lygodium* medium was adsorbed on charcoal and destroyed by ashing, as was the case also for *Pteridium* and *Anemia* factors(2,6). Biological activity of the *Lygodium* medium was further found to be stable to autoclaving for 15 min at a pressure of 15 lb at pH 5 and to boiling for 10 min at pH 2 and 12 (it is possible that some inactivation, just at the verge of detectability, takes place upon boiling at both pH 2 and 12). The *Anemia* factor was stable under these conditions(6), while the *Pteridium* factor differed only by its lability at pH 12(2).

As mentioned earlier, *Pteridium* medium

failed to promote antheridium formation in *L. japonicum* throughout the wide range of applied concentrations (⅓ to 1/30,000 full strength *Pteridium* medium that was active toward prothalli of *O. sensibilis*, *i.e.*, the prothalli used to assay for the *Pteridium* factor, to a dilution of 1:30,000). In the reciprocal experiment 13 out of 16 samples of *Lygodium* medium (active toward prothalli of *L. japonicum* mostly to a dilution of 1:300) were without effect in *O. sensibilis*. Two of 16 samples brought about an antheridial response in *O. sensibilis* if only at the highest concentration (⅓ full strength). These 2 samples were active toward *L. japonicum* to a dilution of 1:300. The remaining sample brought about an antheridial response in *O. sensibilis* to a dilution of 1:10 even though it was active toward *L. japonicum* only to a dilution of 1:100. The last-mentioned sample of *Lygodium* medium was boiled for 10 min at pH 12, under which conditions the *Pteridium* factor was shown to be labile(2). The batch of *Lygodium* medium thus treated failed to bring about an antheridial response in *O. sensibilis* even at the highest concentration. In contrast, its activity toward *L. japonicum*, as tested again by a dilution series, remained unimpaired. This experiment could not be repeated because all subsequently harvested batches of *Lygodium* medium lacked detectable activity toward *Onoclea* prothalli.

Even though a few samples of *Lygodium* medium showed very slight activity toward *O. sensibilis*, the factor controlling antheridium formation in *L. japonicum* would appear to be chemically distinct from the *Pteridium* factor. This is indicated by the unresponsiveness of *Lygodium* prothalli toward *Pteridium* medium even at a concentration 10,000 times higher than that necessary to bring about a minimal response in *Onoclea* prothalli. The selective destruction of the activity of *Lygodium* medium toward *O. sensibilis* upon boiling for 10 min at pH 12 and the indicated variable proportion between the antheridium-inducing activities of *Lygodium* medium toward *O. sensibilis* and *L. japonicum* point in the same direction. The *Lygodium* plants from which spores were obtained grew together with many other fern species, hence the possibility cannot be discounted that the antheridium-inducing activity toward *O. sensibilis* was elaborated by the prothalli of a species other than *L. japonicum* (amount of antheridium-inducing factor produced by a single prothallus of *P. aquilinum* is ample to account for the observed effect(3)).

It was earlier concluded that the *Pteridium* factor is chemically distinct from the substance controlling antheridium formation in *A. phyllitidis*(6). It was of further interest to assay for possible similarity between this *Anemia* factor and the substance that controls antheridium formation in *L. japonicum*.

Lygodium medium failed to bring about an antheridial response in *A. phyllitidis* throughout the wide range of applied concentrations (⅓ to 1/30,000 full strength *Lygodium* medium). The same result was obtained in a dozen repetitions with further batches of *Lygodium* medium which were active toward *L. japonicum* to a dilution of 1:300, occasionally to 1:1,000. In the reciprocal experiment, *Anemia* medium regularly showed activity toward *Lygodium* prothalli. To obtain a minimal antheridial response, however, the *Anemia* medium had to be supplied to the *Lygodium* prothalli at a concentration 10 to 30 times higher than to the *Anemia* prothalli.

Discussion. The results are considered to show that the factor controlling antheridium formation in *L. japonicum* differs from the *Pteridium* factor, *i.e.*, the substance which controls this same process in many species of the fern family Polypodiaceae.

The further question arises whether this *Lygodium* factor is identical with the antheridial factor of *A. phyllitidis*. The need for a higher concentration of *Anemia* medium to bring about a minimal response in *Lygodium* is consistent with this hypothesis if we postulate that this species possesses a greater capacity than *Anemia* to inactivate, or otherwise neutralize, the active substance. In the reciprocal experiment, though, this hypothesis calls for greater activity of *Lygodium* medium toward *Anemia* than toward *Lygodium* itself. Instead, the *Anemia* prothalli were found to be unresponsive to *Lygodium* medium even at the highest applied concentration. The hypothesis that the same substance controls an-

theridium formation in the two species may be retained by invoking complex subsidiary assumptions. It seems more likely, however, that control of this developmental event in *L. japonicum* will be traced to a different factor than in *A. phyllitidis*. If we assume that the 2 substances are similar, then the factor controlling antheridium formation in *Anemia* might itself be effective also in *Lygodium*, provided it is supplied to the latter species at a high enough concentration. This postulate is compatible with the experimental result. In the reciprocal experiment, failure of *Anemia* prothalli to respond to even the highest applied concentration of *Lygodium* medium is also consistent with this hypothesis if we consider that the *Anemia* factor might more easily "fit" the *Lygodium* acceptor molecule than the similar *Lygodium* factor "fits" the corresponding molecule in *Anemia*. It seems quite as likely, however, that the antheridium-inducing activity of *Anemia* medium toward *Lygodium* is referable to a different substance than its activity toward *Anemia* itself. Work now in progress should clarify some of these questions.

It is hoped that these investigations will contribute to the classification of Pteridophytes which remains in a state of considerable fluidity. More important, they should provide information on the mode of evolution within a closely delimited segment of metabolism. Kluyver and Van Niel have directed attention to the similarity, even identity, of many basic biochemical patterns in taxonomically widely separated organisms. The proposed investigation should be of special interest because of its concern with the less frequently considered question: How similar or dissimilar among organisms are the biochemical processes associated with a given developmental event (*viz.* antheridium formation) that we conceive of primarily in morphological terms and which in different groups of ferns proceeds similarly in terms of morphology and is identical in terms of function?

Summary. It is demonstrated that prothalli of *L. japonicum* elaborate, and secrete into the medium, a substance which controls formation of antheridia in this fern species. The antheridium-inducing activity of the medium is stable to boiling for 10 min at both pH 2 and 12. It is adsorbed on charcoal and destroyed by ashing. The results are considered to show that this antheridium-inducing substance differs from the substance which controls the same developmental event in many species of the fern family Polypodiaceae. The results further indicate that the *Lygodium* factor is also chemically distinct from the substance that controls antheridium formation in *A. phyllitidis* which, like *L. japonicum*, belongs to the fern family *Schizaeaceae*. The individuals that first attain the reproductive phase in a gametophyte population of *L. japonicum* give rise to one or 2 archegonia before the first antheridium initial appears. This sequence of sex-organ formation is reversed in the prothalli which subsequently attain the reproductive phase. All prothalli attain the archegonial phase first if they are isolated, one per flask, at an early stage of development. In contrast, all prothalli attain the antheridial phase first if they are all provided with the antheridium-inducing substance at an early stage of development. It is concluded that the more slowly growing individuals in a gametophyte population of *L. japonicum* attain the antheridial phase first because they respond to the antheridium-inducing substance that is elaborated, and secreted into the medium, by the more rapidly growing gametophytes.

I am grateful to Dr. Armin C. Braun for the encouragement he has given this investigation.

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Effect of Atrial Fibrillation on Left Atrial Pressure and Distensibility of Pulmonary-left Heart Vascular Segment.* (26017)

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Volume-pressure relationships for the pulmonary-left heart vascular segment of the dog have recently been reported(1). The experimental preparation utilized in that study has been modified to investigate the effect of atrial fibrillation on this parameter of the cardiovascular system. The results are presented here.

Method. The basic experimental preparation utilized in this study is described briefly here and appears in detail elsewhere(1). The pulmonary-left heart segment was isolated by occluding the pulmonary artery during short periods of cardiac standstill produced by left vagus stimulation. During this interval of ventricular asystole, a measured volume of saline or blood was injected into the left atrium and the change in pressure recorded. When ventricular escape occurred, the tapes occluding the pulmonary artery were removed and the circulation permitted to return to normal. In this manner a number of different injections were made in the same animal.

Atrial fibrillation was produced electrically by stimulating the atrial appendage with a laboratory stimulator. In most experiments atrial fibrillation was produced following injection; in other animals atrial fibrillation was produced before injection was made. In both cases atrial fibrillation was continued until ventricular escape occurred.

Results and discussion. A typical record of left atrial and aortic pressure during an injection of 20 cc of saline into the relaxed left atrium during a short period of ventricular asystole is shown in Fig. 1. Left atrial pressure rose rapidly during injection and remained constant at its higher level until onset of atrial fibrillation. With onset of atrial fibrillation the pressure fell, then was main-

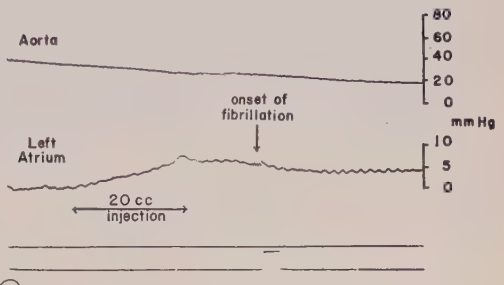

FIG. 1. Record of aortic and left atrial pressure during inj. of 20 cc of saline into left atrium during ventricular asystole. Onset of atrial fibrillation is indicated.

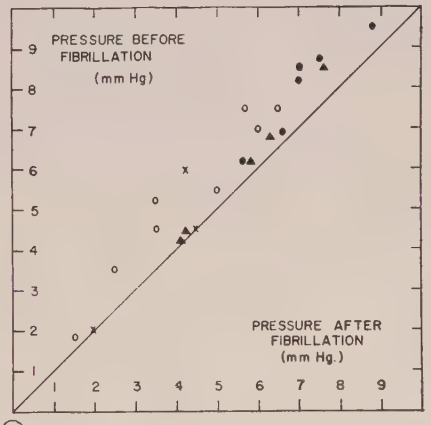

FIG. 2. Relationship between left atrial pressure before and after onset of atrial fibrillation. Each symbol represents a different animal.

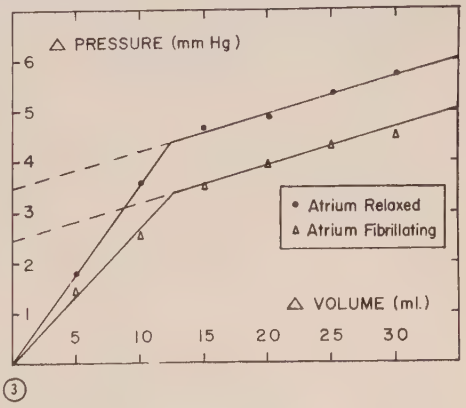

FIG. 3. Volume-pressure relationship shown by plotting change in pressure against change in volume before and after onset of atrial fibrillation.

* This work was supported in part by a grant from Am. Heart Assn.

tained at the lower level for the duration of ventricular standstill. This drop in pressure, while moderate in amount, occurred in all experiments.

Fig. 2 shows pre-fibrillation pressure in the left atrium plotted against pressure after onset of atrial fibrillation for 23 observations in 4 dogs. The data indicate that intracardiac pressure decreased with atrial fibrillation in the order of 1 mm Hg over the entire range of pressure tested. These findings were confirmed in an additional animal where duplicate injections were made over a range of volumes. The first injection of each pair was made into the non-fibrillating atria and the second after atrial fibrillation was produced. In each case the pressure was lower after second injection.

Volume-pressure plots were made for each heart before and after onset of atrial fibrillation by plotting change in pressure against change in volume. All pre-injection pressures agreed within ± 0.5 mm Hg. Fig. 3 shows a typical plot of the data from one animal.†

The shift of the curve toward the volume axis with atrial fibrillation occurred in all experiments. This was an unexpected finding as *a priori* reasoning might suggest that atrial pressure would rise with atrial fibrillation. Displacement of the curve is apparently not due to a change in elasticity of the atrium as the slope of the two curves is essentially the same.

The mechanism responsible for lower intracardiac pressure with atrial fibrillation is not clear. If one assumes that the atria are roughly ellipsoid in shape, it could be further assumed that they would become more spherical in form with fibrillation due to the increase in wall tension. If this change in shape occurred, the internal volume would increase, thereby lowering intracardiac pressure. However, this explanation does not seem entirely adequate. Inspection of the volume-pressure plots indicates that a drop of 1 mm of Hg pressure would require an increase in volume of about 12 cc. A volume change of this magnitude seems too great to be accomplished by variations in atrial geometry. Furthermore, increasing the internal volume would tend to change the slope of the volume-pressure curve which does not occur.

Summary. The effect of atrial fibrillation on left atrial pressure was studied in the living dog during brief periods of ventricular standstill. The data indicate that left atrial pressure decreases with atrial fibrillation in the order of 1 mm Hg. This decrease causes a shift of the volume-pressure plot for the pulmonary-left heart segment toward the volume axis without change of slope. The mechanism of this shift is not clear.

† The break in the plot at a volume change of 12 ml has been described elsewhere(1).

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Ethionine-Induced Resorption of the Rat Conceptus.* (26018)

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Various investigators have reported that amino acid analogues have specific detrimental effects on growth and differentiation of embryonic primordia in the chick(1,2). It has been proposed that the effect is primarily on the protein of the embryonic cell. In light of this, we have established a program to determine effects of amino acid analogues on the rat embryo.

Ethionine, the amino acid analogue of me-

* This project was initiated at Univ. of Oregon; it has been supported by U. S. Public Health grants and by funds from the Graduate School, University of Oregon.

TABLE I. Gross Effects of Ethionine* and/or Methionine* on 12-Day Rat Embryo.

Treatment (total dose)	Total No. rats	Embryonic resorption — No. complete	No. partial	% complete + partial	% embryos reduced in size or resorbed
Saline controls	39	2	3	12.8	5.6
.8 mg ethionine/g body wt	41	23	14	90.2	62.3
.8 mg methionine/g body wt	12	0	0	0	1.4
.8 mg ethionine + .8 mg methionine/g body wt	24	7	6	54.2	26.4

* Nutritional Biochemicals.

thionine, was the first compound chosen for this investigation since its effects on the non-pregnant rat have been studied(3-6). This paper describes gross and histologic effects of ethionine on the pregnant rat with particular reference to resorption of embryos and induced abnormalities of placentae and uteri.

Materials and methods. A total of 116 pregnant albino rats (Sprague-Dawley strain) within a weight range of 230 to 270 g was used. Pregnancies were timed by vaginal smears, the day in which sperm were found being considered as day 0. Animals were treated as shown in Table I. The total dose of ethionine and/or methionine was administered over a 3-day period, starting on day 7. Animals were sacrificed on day 12 of pregnancy. After autopsy, uteri were removed and conceptuses and embryos were measured. Several conceptuses from each animal were fixed in Bouin's solution for histological observations.

Results. A summary of the gross observations is shown in Table I. Resorption of the conceptus was considered as complete when the embryo had been completely resorbed and the placenta either was present as a remnant of tissue or as an implantation site. Partial resorption was indicated by a definite reduction in size of the embryos and presence of large amounts of blood in the uterine lumen. In the calculation of the % embryos reduced in size or resorbed, average litter size of 11 was used for those animals in which complete resorption had occurred.

The pattern of the resorption was apparently similar in ethionine-induced resorption and spontaneous resorption observed in the controls. The sequential destruction of the various regions of the placenta was as follows: The allantoic mesoderm was destroyed 1st (Fig. 1B), followed by the labyrinth and compact layers of the hemoendothelial placenta (Fig. 1C). The embryo was destroyed next as indicated by a loss of cohesion of the cells and an increase in pycnosis, then the yolk sac splanchnopleure disappeared (Fig. 1D), and finally the giant cell trophoblast. The giant cell layer often formed a prominent spherical nest of cells in the center of the conceptus. The decidua basalis was found to be very resistant to breakdown.

There appeared to be no selective effect of ethionine on the embryo, but rather an overall breakdown of embryonic tissue. It was frequently noted that the neural tube had a spiral or wavy appearance, especially noticeable in the late stages of destruction of the embryo.

Discussion. An increase in number of embryonic resorptions and still births in pregnant rats following administration of ethionine has been reported by Lee *et al.*(7). Although the pancreases of the mothers were severely damaged by ethionine injections, those of the fetuses remained essentially normal. Therefore the authors questioned the passage of the ethionine across the placenta. However, when C^{14}-ethionine was administered to pregnant rats 4 days before parturition, the label was isolated from the newborn rat in the form of choline and creatinine(3). The passage of the ethionine across the rat placenta is being investigated in this laboratory by autoradiographic technics. Since it has been shown that ethionine can be incorporated into proteins in the non-pregnant rat (4), it is assumed at present that ethionine exerts its effect by being incorporated into proteins of the embryo and/or placenta and therefore interferes with the normal protein metabolism of these tissues.

FIG. 1. Sections of 12-day pregnant rat conceptuses. A, control. B, C, D, stages in resorption following treatment with ethionine. DB, decidua basalis; E, embryonic remnant; G, giant cell layer; L, placental labyrinth; YS, yolk sac; AM, allantoic mesoderm. Hematoxylin and eosin stain. × 15.

Further evidence for this assumption is the observation that simultaneous administration of methionine and ethionine causes a reduction of approximately 50% in the embryonic resorption and decreased size of the rat embryos. Several workers(5,6) have demonstrated that a higher amount of methionine is required to completely reverse the ethionine effect on the liver. Therefore, it may be that the partial reversal of the resorptive effects of ethionine shown here indicates that the ethionine effect involves competitive incorporation between the analogue and the amino acid.

Summary. A marked increase in resorption

of the embryos and placentae was observed following administration of high doses of ethionine to pregnant rats. A similar dose of methionine had no apparent effect by itself while a combination of methionine with ethionine resulted in about a 50% reduction in the resorptive effect of ethionine. The sequential destruction of the tissues of placenta and embryo associated with the ethionine-induced resorption is described.

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Adrenergic Inhibition and Lethal Effect of Bacterial Endotoxin in Mice. (26019)

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Participation of epinephrine or related substances in the sequelae of bacterial endotoxin administration has been suggested(1-3). Adrenergic blocking agents such as phenoxybenzamine HCl have been used against traumatic shock with some success in laboratory animals, though with less in man. They have been largely ineffective against bacterial endotoxin. Recently several new ways of affecting sympathetic reactivity have been described. The following study tests the ability of some of the presently available types of adrenergic inhibition to alter lethal effects of injected epinephrine and of bacterial endotoxin administration.

Methods. Survival after intraperitoneal epinephrine was determined by the technic of Loew and Micetich(4) and results were evaluated by the method of Litchfield and Wilcoxon(5). Data from selected dose levels were used for comparison in Table I. High doses of epinephrine with low expected survivals were used when protection was anticipated and the reverse when sensitization was indicated. The significance of difference between results from these selected doses was judged by the Rank Correlation method of Wilcoxon(6).* Doses of synthetic l-epinephrine bitartrate were calculated as mg/kg free base. The blocking agents were given as salts, also on a mg/kg basis. Difco crystalline lipopolysaccharide from *E. coli* was used in the endotoxin studies. It was dissolved in pyrogen-free saline and given intraperitoneally on the basis of total mg/mouse (not/kg). Average weight of the male CF-1 mice used in this study was about 20 g. For reference purposes 2 SD_{50}, or 50% survival, doses were determined one hour after epinephrine in normal untreated male CF-1 mice. These were 8.0 (5.41-11.8) and 7.8 (6.45-9.44) mg/kg i.p. respectively. Another series received an intraperitoneal saline blank (0.1 cc/10 g of mouse) 24 hours before epinephrine. SD_{50} here was 7.6 (6.39-9.04) mg/kg i.p. Obviously, the susceptibility of the animals to epinephrine was essentially unchanged. We did find that mice were adversely affected if one intraperitoneal injection followed soon after another. For short term testing we used subcutaneous or oral premedication followed by intraperitoneal epinephrine.

Results. A. *Effects on lethal action of injected epinephrine.* It is recognized that lethal actions of toxic doses of epinephrine are very different from circulatory collapse and shock. However, as a preliminary step in our study we tested the ability of the several

* Kindly computed by Dr. Irving Sher of Smith Kline and French Labs.

TABLE I. Protective Effects of Various Forms of Adrenergic Inhibition against the Lethal Action of Injected Epinephrine.

Condition	Pretreatment	Dose, mg/kg	Pretreat. time, hr	Epin. i.p., mg/kg	1 hr survival living/total and % Test		Control*		Effect†
Peripheral adrenergic									
Excitation									
Block	Phenoxybenzamine	5 p.o.	1	12	10/10	100	2/10	20	+
Sensitization	Cocaine HCl	9 s.c.	1	4	8/20	40	19/20	95	—
Inhibition									
Block	3,4-dichloroisoproterenol	10 "	1	9	4/20	20	5/20	25	—
"Sympatholysis"	TM 10	10 "	1	12	4/20	20	2/10	20	0
	"	10 "	24	12	6/10	60	7/20	35	+
Catechol amine content									
Release	Reserpine	10 i.p.	24	12	7/10	70	7/20	35	+
Accumulation	Iproniazid	100 "	24	3	10/10	100	19/20	95	0

* Physiological saline blanks substituted for pretreatment with the adrenergic inhibitors.
† Effect: +, protected; —, more vulnerable; 0, no effect.

forms of adrenergic inhibition to oppose the acute toxicity of injected epinephrine.

The results of this first phase of our investigation are summarized in Table I. On the left are types of adrenergic influence which are thought to be produced and on the right, survival under these conditions. Saline blanks were run for control. The marked protection by phenoxybenzamine and reciprocal activity of cocaine parallel the well-known autonomic effects of similar doses of these drugs as demonstrated by blood pressure and nictitating membrane responses in anesthetized cats and dogs. Blockade of the peripheral adrenergic excitatory mechanism in the first case and sensitization in the second appear to be indicated. Site of action here is probably the vascular smooth muscle.

An opposite condition is produced by the 3, 4-dichloro analog of isoproterenol.† This substance has been shown to block inhibitory adrenergic responses(7). It also blocks the cardiac stimulant effect of adrenergic substances(8). This latter response is unaffected by blockers of excitatory adrenergic receptors, for example, by phenoxybenzamine. 3,4-Dichloroisoproterenol may well interfere with some of the metabolic processes initiated by epinephrine and which are related to vasodilation rather than vasoconstriction. Ten mg/kg of 3,4-dichloroisoproterenol were given subcutaneously to the mice one hour before the intraperitoneal epinephrine. From the results obtained in various species by the authors cited above such a procedure should establish the characteristic effect of this compound. It did not protect against 9 mg/kg of epinephrine. In fact, 25% of the control animals survived and only 20% of the treated animals were able to withstand the epinephrine. This difference is not significant in the rank correlation test. To check whether this difference was real, a larger number of mice were used and dose response curves were calculated for animals treated as described above and for parallel control groups dosed with saline. These lines were not parallel but this was not surprising. The ED_{50} for saline controls was 6.5 (5.0-8.4) mg/kg epinephrine i.p., while comparable figures for animals pretreated with 3,4-dichloroisoproterenol was 4.3 (2.9-6.3) mg/kg. This difference is significant ($p = 0.074$).* Clearly, pretreatment with this type of peripheral adrenergic blocker has reduced ability of the mice to survive acute lethal effects of injected epinephrine.

A unique type of adrenergic inhibition was recently described by Exley(9). 2,6-Xylyloxyethyltrimethylammonium bromide, or TM 10, was shown to produce some form of blockade of the terminal sympathetic nerve endings. An interference with enzymes on the metabolic pathway to epinephrine and norepinephrine has been suggested. Whatever the mode of action, the net result appears

† Generously supplied by Dr. I. H. Slater, Eli Lilly Co., Indianapolis, Ind.

TABLE II. Protective Effects of Various Forms of Adrenergic Inhibition against Lethal Action of Injected Endotoxin.

Condition	Pretreatment	Dose, mg/kg	Pretreat. time, hr	Lot No.*	48 hr SD_{50} of endotoxin, mg i.p.; values in parentheses are 95% confidence limits		Effect at p .05
					Test	Control†	
Peripheral adrenergic							
Excitation							
Block	Phenoxybenzamine	5 p.o.	1	β	.56 (.42-.74)	.47 (.39-.58)	0
Sensitization	Cocaine HCl	9 s.c.	1	γ	.38 (.32-.46)	.31 (.24-.40)	0 ?
Inhibition							
Block	3,4-dichloroisoproterenol	2 × 10 s.c.	1 & 18	γ	.55 (.47-.64)	.29 (.26-.33)	+
"Sympatholysis"	TM 10	10 s.c.	1	γ	.31 (.24-.41)	.27 (.21-.36)	0
Catechol amine content							
Release	Reserpine	10 i.p.	24	β	.37 (.27-.50)	.47 (.39-.58)	− ?
Accumulation	Iproniazid	100 "	24	β	.44 (.38-.51)	.47 (.39-.58)	0 ?

* Endotoxin lot number.
† Physiological saline blanks substituted for pretreatment with the adrenergic inhibitors.

to be reduced sympathetic reactivity. The term sympatholytic would seem to fit this compound. A dose of 10 mg/kg subcutaneously, which should be effective according to Exley's results, failed to offer protection when given one hour before the challenging dose of epinephrine. If given 24 hours ahead more treated animals survived than did saline controls. However, the difference was not significant by the rank correlation method. If active at all TM 10 is certainly far less effective than phenoxybenzamine in this test.

A very different type of adrenergic influence is produced by reserpine. We found little or no evidence of peripheral adrenergic blockade when this alkaloid was tested in cats and dogs as noted above. At the same time it markedly reduces excitability of the peripheral vasculature and is widely used in human hypertension. Recently a number of investigators have attributed its inhibition of vascular excitability to release of catechol amines such as epinephrine and norepinephrine from associated chromaffin tissue. In our series of survival studies the effectiveness of this type of adrenergic inhibition was evaluated against acute lethal effects of epinephrine injected into mice. The results are included in Table I. Reserpine increased an expected survival of only 35% after 12 mg/kg of epinephrine to 70%. This difference is significant. In the series of over a hundred mice from which these data were taken more than half the animals survived at all dose levels up to a dose of 30 mg/kg of i.p. epinephrine. In another experiment an SD_{50} of 198 (158-247) mg/kg of epinephrine was obtained about 6 hours after an intraperitoneal dose of 10 mg/kg of reserpine. This is nearly 25 times as high as the control. Clearly, reserpine provided some form of protection in our experiments. In contrast, Luduena *et al.* (10) found no protective effect in rats after pretreatment with reserpine 1 mg/kg, but their pretreatment time was only 3 hours. Failure of iproniazid to influence toxicity is apparent in the table. With a graded series of epinephrine doses an SD_{50} of 8.2 (6.7-10.0) mg/kg of epinephrine was obtained 24 hours after intraperitoneal administration of iproniazid 100 mg/kg. This is almost the same as the normal and saline control SD's mentioned earlier. The implication is that the protective effect of reserpine does not result from release of catechol amines. Possibly the direct smooth muscle depression described by Gillis and Lewis(11) may be responsible for the observed protection. The findings are at variance with results of Borowitz and North(12) who found augmented toxicity on the part of epinephrine after iproniazid pretreatments. They stress the importance of a slow, prolonged administration of epinephine. They also used 24 hour survival. These 2 factors may explain the difference between their findings and ours.

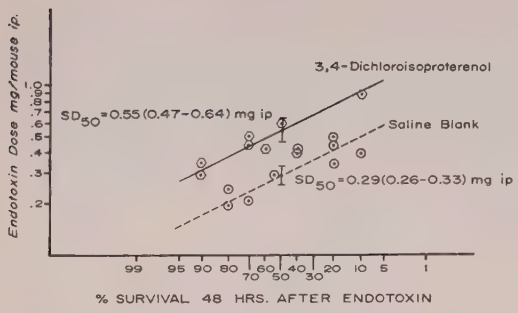

FIG. 1. Dose-response curves including SD_{50} doses and their 95% confidence limits.

B. *Survival after bacterial endotoxin administration.* Experiments with adrenergic blocking drugs such as phenoxybenzamine have offered little promise of clinical usefulness when employed against bacterial endotoxins. Some prolongation of survival time has been obtained, but lethal outcome was not materially altered. It was therefore of considerable interest to us to test these newer forms of adrenergic inhibition against pure crystalline endotoxin. Doses and pretreatment times used were considered adequate to establish the particular type of block or inhibition characteristic of the compound being studied. The results of this series of tests are summarized in Table II.

Blockade of the excitatory adrenergic receptors by phenoxybenzamine has not provided a degree of protection which is significant at p 0.05. Like results were obtained after sensitization of these receptors with cocaine. Somewhat higher doses were required to produce a 50% mortality in each case and low order protective effects are possible. A useful degree of protection is not apparent.

In the case of the next compound, 3,4-dichloroisoproterenol, however, survival was significantly increased. This substance blocks peripheral adrenergic inhibition.

The sympatholytic compound TM 10 seems to offer no protection, at least as it was used in our experiment. Additional tests with 24 hour pretreatment time were also negative. Catechol amine release by reserpine, or accumulation after iproniazid, did not improve survival.

Discussion. The principal point in the foregoing account where questions might be raised is in the matter of dosage and pretreatment time. In the case of phenoxybenzamine and cocaine the obvious results in Table I verify their effectiveness at doses and times employed. Their peripheral site of action in the vascular smooth muscle excitatory system does not seem to be germane to the problem of protection against endotoxin.

The other substances affecting excitatory mechanisms also failed to offer protection. Thus TM 10, which should reduce responsiveness of terminal sympathetic nerve endings, did not increase survival significantly. The dose used was reported by Exley(9) to cause marked relaxation of the nictitating membrane in cats. The effect came on within an hour and lasted more than 24 hours. Reserpine, by depleting catechol amines from chromaffin tissue, reduces overall reactivity of efferent sympatho-adrenal nervous pathways. It did not protect the mice against endotoxin. Six and a half mg/kg of reserpine largely depleted rabbits' ears of catechol amines in 48 hours according to Burn and Rand(13). Shimamoto *et al.*(14) report significant protection of rabbits against *Shigella* endotoxin by doses of reserpine ranging from 1.0 to 5.0 mg/kg. Our dose in Table II is high but smaller doses (1, 3 or 5 mg/kg) also failed to protect. The deleterious effect of the 10 mg/kg dose suggested in Table II may result from fluid loss and poor alimentation. Finally the reciprocal effect of catechol amine accumulation brought about by monoamine oxidase inhibition on the part of iproniazid also had no protective action. The dose of 100 mg/kg should allow a considerable build-up of epinephrine and norepinephrine in 24 hours according to Pletscher(15).

The one case of clear cut protection in Table II is that of 3,4-dichloroisoproterenol. Fig. 1 represents a dose-response curve obtained with mice pretreated with this compound, 10 mg/kg SC, 1 hour before and 18 hours after graded endotoxin doses. This is a very interesting finding. Moran and Perkins(8) reported that this substance blocked cardiac effects of epinephrine. These may be metabolic effects rather than muscular excitant effects involving receptors according to

Mohme-Lundholm(16). Carbohydrate metabolism may be involved.

Summary. 1) Protective effects of various types of adrenergic inhibition have been tested against lethal action of injected bacterial endotoxin in mice. 2) Preliminary experiments substantiated abilities of phenoxybenzamine to block lethal effect of injected epinephrine and of cocaine to augment its toxicity. 3,4-Dichloroisoproterenol also increased lethal effect of epinephrine. TM 10, or 2,6-xylyloxyethyltrimethylammonium bromide had no effect acutely but offered low order of protection when given 24 hours before epinephrine. Reserpine also protected when given 24 hours in advance. Iproniazid was without effect. 3) Against bacterial endotoxin from *E. coli* the only compound to give significant protection was 3,4-dichloroisoproterenol. This substance has been shown by previous workers to block inhibitor and vasodilator effects of epinephrine and related catechol amines. Such effects are probably metabolic in nature and may concern carbohydrate metabolism.

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Placental Transfer and Fetal Tissue Uptake of Mg^{28} in the Rabbit.* (26020)

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The factors regulating the metabolism of magnesium are obscure and very little is known about the placental transfer of this ion or its uptake in the fetal tissues. The purpose of the present study in rabbits was to investigate, by use of a radioactive isotope of magnesium (Mg^{28}), the maternal-to-fetal transfer of magnesium and uptake of the isotope by various maternal and fetal tissues.

* This work was supported in part by contract between U. S. Atomic Energy Comm. and the University and in part by a grant-in-aid from Playtex Park Research Inst.

Material and methods. Pregnant domestic albino rabbits, between 28 and 30 days of gestation, were anesthetized with sodium pentobarbital given intravenously in a dosage of 40 mg/kg of body weight. After a tracheotomy had been performed, the carotid artery was cannulated and an indwelling polyethylene catheter, connected to a reservoir of heparin, was inserted.

Mg^{28} was then injected into the marginal vein of the ear as a solution of magnesium sulfate, prepared according to a method previ-

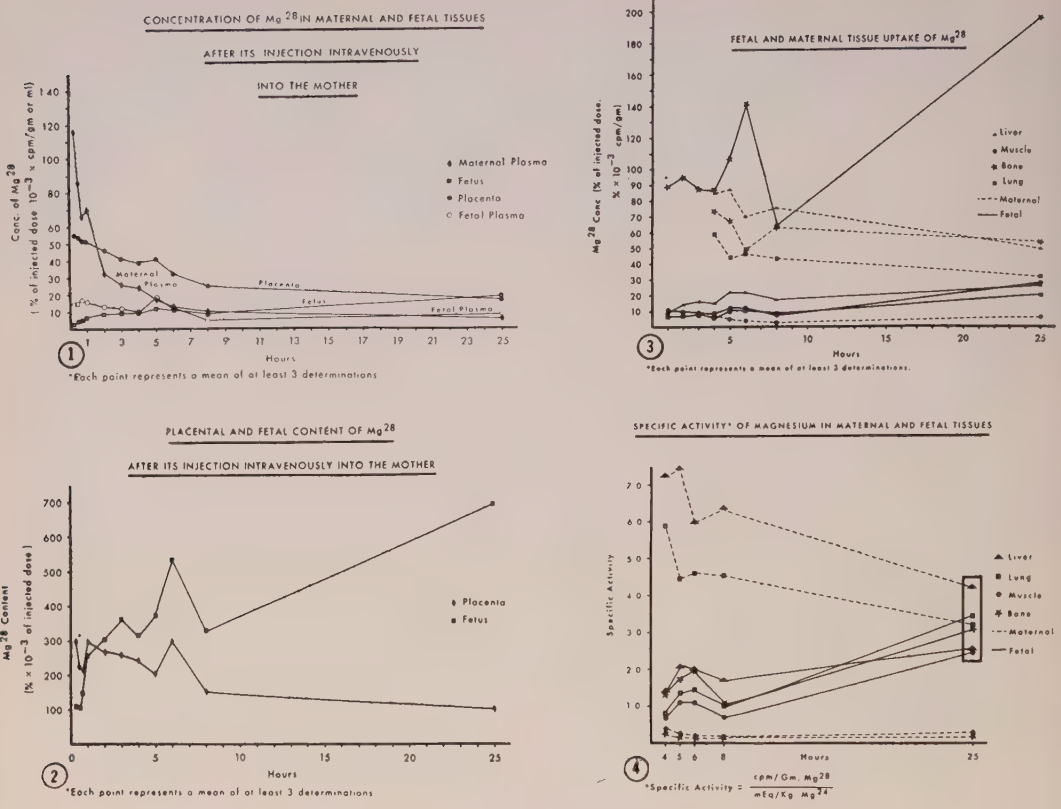

FIG. 1-4.

ously described(1); 20 μc of Mg^{28} was contained in 2 meq of stable magnesium.

Abdominal cavity was exposed by a midline incision. At intervals varying from 7 minutes to 26 hours after injection of Mg^{28}, a fetus and its placenta were removed from the uterus. A wide V-shaped incision was made over the thorax of the fetus and blood was obtained by cardiac puncture with a 20-gauge hypodermic needle attached to a heparinized syringe. Simultaneously, with withdrawal of fetal blood, a maternal blood specimen was obtained from the carotid artery. At termination of each experiment, the mother was killed by air embolism and samples of tissues were obtained for assays of radioactivity and magnesium content.

Serum and tissue magnesium determinations were performed by the molybdivanadate method for phosphate(2).

Radioactivity assay. Samples of plasma and tissues were assayed for gamma ray activity in a well-type scintillation counter. Total Mg^{28} content of each fetus and placenta was determined; samples of liver, kidney, skeletal muscle, tibia and femur, and skull were then removed from each fetus and their concentrations of radioactivity determined separately. All results are expressed as percentages of total administered dose of radioactivity.

Results. A total of 86 fetuses and their respective placentas were obtained from 11 pregnant rabbits. Clearance of Mg^{28} from the maternal circulation, its concentration in placenta and fetus, and appearance of Mg^{28} in fetal circulation are summarized in Fig. 1.

Changes in concentration of Mg^{28}. Concentration of Mg^{28} in maternal plasma decreased rapidly during the first 2 hours; thereafter, the decline was slower, but progressive. Concentration of Mg^{28} was initially lower in the placenta than in the maternal plasma, but within 2 hours, placental concentration became higher and remained higher thereafter.

The progressive decrease of Mg^{28} in the placenta paralleled that in the maternal plasma. Concentration of Mg^{28} in the fetus was initially lower than that in maternal plasma or placenta; it increased gradually, however, and at 24 hours exceeded both maternal and placental concentration of the isotope.

Changes in Mg^{28} content. Average fetal content of Mg^{28} increased progressively (Fig. 2). Between the first and second hour it surpassed the placental content and remained higher thereafter. As fetal content of Mg^{28} increased, that in the placenta decreased progressively.

Tissue distribution of Mg^{28} (Fig. 3). Of all the *maternal* tissues studied, uptake of Mg^{28} was lowest in the muscle. Successively higher concentrations of Mg^{28} were found in lung, long bone, liver and kidney. Concentration of Mg^{28} in all maternal tissues tended to decrease progressively with time as concentration in the fetal tissues increased.

Relative concentrations of Mg^{28} in *fetal* tissues remained consistently in the following order: (1) lung (lowest), (2) muscle, liver and kidney (intermediate) and (3) bone (highest). Comparison of tissue concentrations of Mg^{28} in mother and fetus at various time intervals revealed that concentrations of Mg^{28} in the kidney, lung and liver were higher in the mother than in the fetus. Mg^{28} concentrations in fetal muscle and bone, however, were much higher than maternal concentrations.

The maternal and fetal *tissue contents* of magnesium are summarized in Table I. Magnesium content of all the tissues studied was lower in the fetus than in the mother, the most notable difference being bone.

Specific activities of maternal and fetal liver, lung, muscle and bone are summarized in Fig. 4. By 26 hours, all values, except those for maternal bone and muscle, appeared to reach a constant level.

TABLE I. Fetal and Maternal Tissue Content of Magnesium.

Tissue	Muscle	Kidney	Liver	Lung	Bone
Mother	19.8*	18.5	11.7	10.0	298.1
Fetus	10.8	11.2	10.4	7.9	62.9

* meq/kg (wet wt) of tissue.

Comment. When Mg^{28} was injected intravenously into pregnant rabbits, its clearance from the blood stream was similar to that previously observed in healthy young adult rabbits(3). Its distribution in tissues of the mothers was also similar to that in nonpregnant animals, except for slower uptake in bone and muscle of pregnant rabbits. Previous studies have shown that, in pregnancy, magnesium is mobilized from maternal tissues (4). In addition, bone uptake of Mg^{28} tends to decrease with age. Both of these factors probably contributed to the low uptake of Mg^{28} by maternal bone and muscle.

The observation that placental concentration of Mg^{28} at 2 hours was higher than concentration in maternal plasma suggests that the placenta actively concentrates magnesium. Because of the low specific activity of the Mg^{28} available for this study, the mother was subjected to a slight magnesium load. This maternal loading may have affected the placental concentration of Mg^{28}.

Comparison of tissue magnesium content in mother and fetus shows a higher concentration of untagged magnesium in all maternal tissues studied. In spite of the lower magnesium concentration of fetal tissues, Mg^{28} uptake was rapid and increased progressively. The fact that the specific activities of all fetal tissues and of maternal liver, kidney and lung approached a constant value at 24 hours suggests that Mg^{28} was at equilibrium with the untagged element in these tissues by this time. It is of interest that the specific activity of fetal muscle and bone was considerably higher than that of the respective maternal tissues. Previous studies support the interpretation that rabbit fetuses at this stage of development are rapidly increasing in bone and muscle mass and, hence, turnover of magnesium in these tissues is more rapid than that in the respective maternal tissues.

Our results show that Mg^{28}, injected intravenously into the mother near term, rapidly crosses the placenta and, because of the more rapid growth of the fetus, is concentrated in the fetal tissues. These results further suggest that rate of uptake of magnesium by various tissues is related to anabolic activities of the cells involved. In comparison with the

cellular exchange of potassium, for instance, transport of magnesium across the cell membrane is a relatively slow process.

Summary. Mg^{28} was injected intravenously into 11 pregnant rabbits between 28 and 30 days of gestation. Fetuses and placentas were removed at intervals ranging from 7 minutes to 26 hours. Maternal tissue uptake of Mg^{28} resembled that previously found in nonpregnant young adult rabbits, except that uptake in bone and muscle was slower. Concentration of Mg^{28} in the placenta rapidly rose above the maternal plasma level. Specific activity of magnesium in all fetal tissues studied reached a fairly constant value by 26 hours. Magnesium turnover in tissues of the fetus *in utero,* especially in bone and muscle, is considerably more rapid than that in the respective tissues of the mother.

Mg^{28} was supplied by Brookhaven Laboratory on allocation from U. S. Atomic Energy Comm.

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Systemic and Coronary Hemodynamic Effects of 2 Amino-4-Methyl Pyridine.* (26021)

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2 amino 4 methyl pyridine, also known as RA 1226, W 45, and Ascensil® is a sympathomimetic agent which has been reported to have a marked pressor effect in the experimental animal(1,2). At higher dosage levels (1-3 mg/kg) a moderate positive inotropic effect is reported(3). Furthermore the agent is reported to have a long period of action and no tachyphylaxis(2). The present study concerns the acute systemic and coronary hemodynamic effects of this agent.

Material and methods. The study was done in 10 mongrel dogs weighing between 19 and 26 kg. Anesthesia was secured by administration of 3 mg/kg of morphine sulphate subcutaneously followed one hour later by 0.25 ml/kg of a 50/50 mixture of Veterinary pentobarbital and Dial-Urethane.† During the ensuing 1 hour after administration of the anesthetic cardiac catheters were maneuvered fluoroscopically into the pulmonary artery, coronary sinus and the right atrium and Cournand needles were placed in the femoral arteries. Pressures were recorded by Statham Strain gages on the Gilson Macro-polygraph with means determined by electrical integration. PH was determined on whole blood with the Cambridge model R pH meter. Cardiac outputs were determined by the Fick principle simultaneously with the Hamilton indicator dilution method. Coronary blood flow was measured by the N_2O method. Blood gas analyses were done by the method of Van Slyke and Neill whereas expired air was analyzed by the method of Scholander. Formulae used for calculations are those used traditionally in measurements of coronary blood flow(4), ventricular work and vascular resistance were calculated by the usual formulae(5) without subtracting atrial pressure

* This work was supported in part by grants from Nat. Heart Inst., U. S. P. H. S., Wisconsin Alumni Research Foundation, and Wisconsin Heart Assn.

† Veterinary nembutal contains 60 mg pentobarbital/ml. Dial-Urethane was supplied by CIBA Pharm. Products, Summit, N. J., and contains Dial 100 mg/ml, Monoethylurea 400 mg/ml, and Urethane 400 mg/ml.

TABLE I. Hemodynamic Effects of Ascensil.

Parameter	Before ± SEM	After ± SEM	% change	p value <
Heart rate	88 ± 9.4	54 ± 8.5	−38.7	.001
Mean systemic art. pres.*	126 ± 5.3	149 ± 5.8	+18.3	.001
" pulmonary art. pres.*	12 ± 1.0	13 ± .9	+ 8.3	.3
" right atrial pressure*	1.6 ± .31	3.1 ± .52	+93.8	.001
Body O_2 consumption	116 ± 5.6	112 ± 6.4	− 3.4	.1
" R. Q.	.82± .02	.82± .02	—	—
Arterial O_2 content†	18.8 ± .5	18.2 ± .5	− 3.2	.2
A − VO_2 difference†	4.4 ± .2	6.7 ± .3	+52.3	.001
Art. − cor. sinus O_2 difference†	12.1 ± .9	12.3 ± .8	+ 1.7	.7
Cardiac output (l/min.)	2.7 ± .1	1.7 ± .1	−37.1	.001
Left ventricular work‡	4.7 ± .4	3.4 ± .2	−14.9	.01
Right " " ‡	.5 ± .06	.3 ± .04	−40.0	.01
Total periph. resist. (cg units)	3803 ±223	7329 ±274	+92.7	.001
" pulm. " (")	358 ± 33	642 ± 77	+79.3	.001
Coronary blood flow§	106 ± 7.2	78 ± 5.5	−26.4	.01
Cardiac O_2 consumption§	12.4 ± .6	9.4 ± .5	−24.2	.001
" R. Q.	.84± .01	.82± .03	− 2.4	.7
Coronary vascular resist. (units)	1.24± .08	1.99± .15	+60.5	.001
Index of efficiency‖	.38± .02	.37± .02	− 2.6	.3

* Expressed in mm Hg. † Expressed in ml/100 ml of blood. ‡ Expressed in kg meters /min. § Expressed in ml/100 g myocardium/min. ‖ kg meters of left ventricular work done/min. divided by ml of O_2 used/100 g of left ventricle/min.

since left atrial pressure was not determined. The drug was administered with a constant infusion apparatus at a rate of 4 mg/min starting 5 minutes before the second determination of cardiac output and continuing throughout measurement of both output and flow. One animal received slightly less drug because of technical difficulties with the infusion apparatus but the results were similar to the others and the data are included.

Results. Results are summarized in Table I. Heart rate was decreased by 38.7% ($p<0.001$) whereas mean arterial blood pressure was increased by 18.3% ($p<0.01$). Pulmonary arterial blood pressure was not increased significantly, but mid right atrial pressure increased 93.8% ($p<0.001$). Neither the minute volume of respiration, oxygen consumption, carbon dioxide excretion or body respiratory quotient changed significantly. Although arterial oxygen content did not change significantly there was a 20.7% decrease ($p<0.001$) in mixed venous oxygen content with a 52.3% increase ($p<0.001$) in the arterio-venous oxygen difference. Similarly the mixed venous carbon dioxide content did not change, but arterial carbon dioxide content decreased with a significant increase in the mixed venous-arterial carbon dioxide content (+51.5%, $p<0.001$). The coronary sinus blood oxygen content did not change, nor did the arterial coronary sinus oxygen difference. Neither femoral arterial nor coronary sinus blood pH changed significantly. Hemoglobin and hematocrit were identical in the first and second studies. Cardiac output determined by the Fick principle decreased 37% ($p<0.001$) and output as measured by the Hamilton indicator dilution method decreased the same amount. Left ventricular work decreased 14.9% ($p<0.01$) and right ventricular work decreased 40% ($p<0.01$). At the same time total peripheral resistance increased by 92.7% ($p<0.001$) and pulmonary vascular resistance increased 79.3% ($p<0.001$). Coronary blood flow decreased (−26.4%, $p<0.01$). Myocardial oxygen consumption per 100 g decreased by 24.2% ($p<0.001$), but cardiac respiratory quotient did not change. Coronary vascular resistance increased 60.5% ($p<0.001$) and the calculated index of efficiency remained unchanged.

Discussion. Previous investigation has shown that Ascensil® produces an increase in cardiac output in both compensated and decompensated rat heart-lung preparations(1). No chronotropic effect was shown, but a positive inotropic effect was demonstrated(1,2,3). Intravenous administration of this agent in the dog produced bradycardia and an increase in blood pressure presumed to be due to increased peripheral vascular resistance(2). No

tachyphylaxis occurred in contrast to many of the sympathomimetic series of drugs(2). Perfusion of the coronary vessels by the drug was associated with a variable response as determined by the Rein flow meter(3).

The present investigation has shown a remarkable increase in systemic, pulmonary, and coronary vascular resistance after administration of 2 amino-4-methyl pyridine. The bradycardia is presumed to be vagal secondary to increased peripheral vascular resistance and elevated systemic arterial pressure, since it can be blocked by atropine.

Contrary to the effects of some sympathomimetic agents no increase in body or cardiac oxygen consumption was produced by administration of 2 amino-4-methyl pyridine. The reduction in cardiac output was sufficient to decrease left ventricular minute work even though calculated stroke work and systemic arterial pressure were increased. In line with reduced cardiac work and slower cardiac rate, coronary blood flow and myocardial oxygen consumption decreased. That there was no significant change in cardiac efficiency, seems particularly significant in view of the decrease in cardiac output, since work against increased pressure is reported to be more costly to the myocardium than work induced by increased flow(6). This maintenance of efficiency is most probably attributable to the decrease in cardiac rate since it has been shown that cardiac rate is inversely related to efficiency(7).

Conclusion. 1. The systemic and coronary hemodynamic effects of Ascensil®, (2-amino-4-methyl pyridine) also known as RA 1226 and in the German literature as W 45, has been investigated in 10 mongrel dogs. 2. Its administration has been associated with a marked increase in peripheral, pulmonary and coronary vascular resistance accompanied by an increase in peripheral arterial and right atrial pressure. 3. Considerable reduction occurred in cardiac output, coronary blood flow and myocardial oxygen usage per 100 g per minute, subsequent to its administration. 4. Calculated cardiac efficiency, body oxygen consumption, total body respiratory quotient and cardiac respiratory quotients were unchanged.

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Glyceride Content of Human and Canine Red Blood Cells. (26022)

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During studies on clearance of intravenously administered fat emulsions, chemically direct measurements were made of the tri-, di-, and monoglyceride content of washed red blood cells. Because of some unusual and interesting observations, it was believed desirable to report the results of these studies.

Methods. Plasma was separated from the cells of heparinized whole blood samples in a 5°C refrigerated centrifuge. The cells were washed with an equal volume of 0.9% NaCl solution, recentrifuged at 2260 g for 30 min-

TABLE I. Glyceride and Protein Bound Fatty Acid (NEFA) Content in Micromoles per Liter of Washed Red Blood Cells of Dogs (n = 8) and Human Males (n = 9).

	Dog		Human	
	Mean	S.D.	Mean	S.D.
Triglyceride	199	55	245	90
Diglyceride	413	120	247	50
Monoglyceride	124	55	149	48
NEFA	184	52	357	89
Total—μM/l	920	152	998	145
μeq/l	1,731	301	1,735	305

utes, and the liquid decanted. The buffy coat was discarded and the red cells were washed 3 more times as above. The washed red cells were then hemolyzed in 2 volumes of distilled water with mechanical agitation. When complete hemolysis had occurred, 1 ml portions were immediately placed into appropriate extraction solutions (chloroform : zeolite for glycerides; heptane : isopropanol : sulfuric acid for non-esterified fatty acids or NEFA). Tri-, di-, and monoglycerides were separated on small silicic acid columns; after alkaline hydrolysis and periodate oxidation, the glycerides were measured by the color developed with chromotropic acid(1). NEFA was determined by the method of Dole(2); lactic acid and acidic phospholipids are possibly included in this fraction(3).

Results and discussion. Tri-, di-, monoglyceride, and NEFA levels of human and canine red blood cells are given in Table I. Whereas in cells of both species the content of tri- and monoglycerides is similar, the dog cells have considerably more diglyceride and less NEFA than human red cells. In plasma of dogs fasted 20 hours total glyceride level matches that of the cells (Fig. 1); however, 75% is triglyceride and proportionately much less di- and monoglyceride occurs in the plasma than in the red cells. Human males fasted for 12 hours had twice as much total glyceride in blood serum as in red cells. Since the cell and serum contents of mono- and diglycerides are similar, it appears that almost 4 times as much triglyceride occurs in a given volume of fasting blood serum as in the red cells of humans.

Amounts of glycerides and NEFA in red blood cells fluctuate within the limits indicated by the standard deviations shown in Table I. These are relatively fixed levels and are not influenced by great elevation in fat content of the plasma. Thus, in humans 4 hours after meals containing 50 and 100 g of corn oil or cocoanut oil and also in dogs 8 to 30 minutes after being given intravenous fat emulsion, the washed red blood cell glycerides and NEFA were not significantly different from those in the fasting state.

Previously reported determinations of neutral fat in erythrocytes employed indirect procedures in which phospholipids and cholesterol were deducted from total lipid to obtain the neutral fat. By this means Erickson and associates found 51 mg per 100 g of human red cells and from 40 to 69 mg of neutral fat in 100 g of ox, sheep, or chicken red cells(4). If 277 is assumed to be the average molecular weight of the fatty acids of red cell glycerides as can be calculated for human plasma(5),

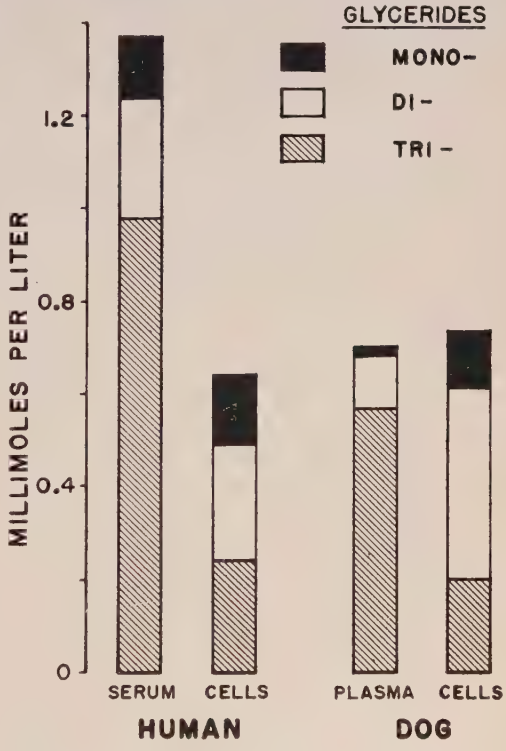

FIG. 1. Glyceride contents of red blood cells compared with that of serum and plasma in fasting humans and dogs. Human serum data represents mean values of 21 male subjects, ages 20-29 yr. Dog plasma values are mean values of 6 determinations in 3 dogs.

the present data would indicate 42 mg of total glycerides per 100 ml of cells.

Hirsch recently showed that complex mixtures of lipids can be separated quantitatively by sorption and elution from silic acid columns(6). We have modified his method so that small quantities of tri-, di-, and monoglycerides can be extracted from 1 ml blood samples, and a direct chemical determination of glyceride glycerol is then performed(7).

Ponder's experience with the cytochemistry of erythrocytes of diverse size and shape led him to express the composition in terms of number of various molecules per cell(8). Taking the volume of a dog erythrocyte as 67 μ^3 and that of the human as 90 μ^3, canine erythrocytes have 0.37×10^8 and human erythrocytes have 0.54×10^8 molecules of glycerides plus NEFA per cell. This is about one-sixth the number of hemoglobin molecules. On the basis of surface areas of 127 μ^2 and 168 μ^2, the glyceride and NEFA fatty acid residues would occur about once in each 175 square Angstroms of cell surface. A surface distribution of this intensity can be compared with the 520 $Å^2$ assigned to one molecule of tetradecylsulfate for production of spheres; "lysis occurs when there is only one molecule per 44 $Å^2$ of cell surface as a minimum"(9).

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Failure to Demonstrate Anti-Hormonal Antibodies in Rats after Maximal Response to Daily Administration of Growth Hormone.* (26023)

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Nearly pure growth hormone (GH), but not pure GH, produced antibodies in rabbit and guinea pig without adjuvant(1). Other reports cited in(2) are open to question because of lack of rigor of proof of purity of GH employed. Pure GH with Freund's adjuvant produces GH-inactivating antibodies(2). Freund's adjuvant can apparently cause autoantibody formation(3), and therefore may have hapten action. Adult Norway rats treated with highly purified GH or GH-containing extracts eventually cease growing at a given dosage per unit weight irrespective of how great this dosage(4). This phenomenon (plateauing) does not result from diabetes (5), ageing of the rat(6), altered steroid production(7), or from altered cardiac output or metabolic rate(8). In the present investigation, serum from plateaued GH-treated giant rats was assayed for anti-GH activity to determine if GH antibodies, or antihormones, are involved in plateauing.

Materials and methods. Eight female Long-Evans rats, plateaued after 248 daily s.c. injections of a pH 10.5 extract of bovine anterior pituitaries(5), at a dose representing 400 mg anterior lobe tissue/kg body weight/day (equivalent to 12.8 mg GH/kg/day), served as source of "treated serum." These rats had attained a mean body weight of 633 g (2.4 times that of their controls). During the first 40 days of treatment, they gained an

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TABLE I. Effect of Serum from Plateaued Rats on Growth Response to GH-Containing Extract.

No. rats	Daily dose	10-day wt gain (g)
5	25 mg* + 1.75 ml treated serum	31.2 ± 3.3†
5	25 " + 1.75 " control "	34.4 ± 3.5
10	25 " + 1.75 " saline	30.0 ± 1.3
8	25 " undiluted	35.7 ± 2.5
10	10 " "	13.7 ± 2.2
17	None	1.9 ± .8

* Dosage is expressed as wet wt of anterior lobe tissue represented in the daily dose; 25 mg of extract is equivalent to 0.8 mg pure GH by 10-day normal rat wt gain assay.

† Stand. error of mean.

average of 113.5 g ± std. error of 4.0 g over their controls, compared to 4.0 ± 4.5 g over controls the last 40 days. These rats were exsanguinated under ether 12 hr after last injection and their serum incubated with aliquots of above pituitary extract in ratio of 1.75 ml serum (8.5% calculated serum of average donor) to 25 mg of extract (10% of daily dose per donor rat during last 40 days of treatment). After 30 min incubation at 10°C, pH 9.0 (no turbidity, possibly due to pH), the mixture was divided into injection vials and stored at –30°C until 30 min prior to injection. Mixtures of same volume of serum from control rats and extract, and of normal saline and extract were prepared and handled identically. These 3 mixtures and 2 dose levels of undiluted extract were assayed for growth potency by daily s.c. injections for 10 days in 5 groups of normal 7-mo-old Long-Evans female rats.

Results. Growth response to the GH extract was not altered significantly by "treated serum" (Table I). The much smaller response of the 10 mg group compared to any of the 25 mg groups indicates that appreciable attenuation would have been detectable. Arthus type reaction with edema, inflammation and some necrosis at site of injection occurred in all 5 rats receiving both extract and treated serum and in no rat in other groups, indicating antibodies to some of the proteins of the extract.

Discussion. The procedures employed do not detect GH-inactivating antibodies in serum of rats plateaued on high dosage of a GH-containing extract. It is conceivable that donor rats were producing GH-inactivating antibodies but at a rate such that the daily GH dosage cleared the antibody from serum for 12 or more hours. It is also possible that plateauing may be due to fixed GH-inactivating antibodies.

Summary. Serum from female rats plateaued while on treatment with a GH-containing extract (after reaching 2.4 times normal adult female size) was ineffective in attenuating GH response of previously uninjected assay rats.

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Spleen Adenosine Deaminase and Guanase Activities after Whole-Body X-Irradiation of Rats. (26024)

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It has been shown(1) that the activities per mg of nitrogen (specific activity) of 2 purine-metabolizing enzymes of liver homogenate, adenosine deaminase and inosine phosphorylase, were unchanged from 16 hours to 10 days after rats were given 600 r of whole-

body X-irradiation. At the same time, it was pointed out that specific activity of inosine phosphorylase of spleen homogenate was increased about 50% although total organ activity of the enzyme was significantly lowered. Other spleen enzymes recently studied in this laboratory have been ribonuclease(2) and DPNH cytochrome *c* reductase(3) both of which exhibited increases in homogenate specific activity, but to varying degrees, 13 to 16 hours after irradiation. As an extension of the investigation of enzymes involved in metabolism of ribonucleic acid and purines, this paper reports the effect of 600 r of whole-body radiation on rat spleen adenosine deaminase and guanase and discusses the interpretation of these results as well as previous data obtained with spleen enzymes.

Methods. A. Irradiation procedure. Three experiments were carried out with male rats of the Wistar strain obtained either from a commercial breeder (Exp. I and II) or directly from Wistar Inst. (Exp. III). A total of 40 animals (190-200 g) were studied for enzyme activity in Exp. I, 24 (120-160 g) in Exp. II, and 24 (185-210 g) in Exp. III. In the first 2 experiments, the rats were given a single dose of 600 r of 240 KV X-rays filtered through 1.0 mm Cu and 1.0 mm Al at an average rate of 66.8 r/minute. In the third experiment, 600 r of 220 KV X-rays were delivered through 0.25 mm Cu and 1.0 mm Al at an average rate of 92.6 r/minute. Target distance was 50 cm in all experiments. The animals were irradiated 2 at a time in a large crystallizing dish covered with a sheet of light cardboard. Food was withheld from all animals for 24 hours after irradiation, then controls were pair-fed against X-rayed rats with a stock commercial diet. Water was supplied *ad libitum*.

B. Tissue preparation and enzyme assays. Spleens were removed from the rats under ether anesthesia after exsanguination by heart puncture, washed free of adhering blood, and homogenized 1:10 with cold glass-distilled water in a Ten Broeck homogenizer. For the adenosine deaminase tests, the homogenates were further diluted to 1:250 in water. Deaminase activity was assayed spectrophotometrically by following the decline of the 265-mμ peak of adenosine with time (adenosine$\rightarrow$inosine + NH_3). Components of the reaction mixture, in their order of addition to cuvettes, were 0.1 or 0.2 ml of homogenate, 0.5 ml of 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4, and 0.3 micromole of adenosine. Readings were taken at 1-minute intervals for 6 minutes.

Before studying effect of irradiation on guanase activity, a number of preliminary experiments were carried out, for although the presence of this enzyme in rat spleen has long been known, no data were available concerning application of Kalckar's spectrophotometric technic(4) to normal spleen guanase assays. Guanase activity of rat spleen homogenates (1:50 in water) was followed by the rate of decline of the 250-mμ peak of guanine (guanine$\rightarrow$xanthine + NH_3). As a check on the reaction under study, xanthine formation was determined by fortifying the reaction mixtures with purified milk xanthine oxidase which converted xanthine stoichiometrically to uric acid. The latter was measured by the appearance of its 292-mu band. Since absorbancy changes at 250 mμ for consecutive time intervals were not always regular, due probably to settling of particles occasioned by use of more concentrated homogenates than in the adenosine deaminase test, it was decided to study guanase activity in the soluble fractions prepared from spleens of normal and X-rayed rats. This was possible, for in aqueous homogenates centrifuged at 56,550 $\times$ g for 45 minutes, about 90% of total guanase activity was recovered in the supernatant fluid to give a 2-fold concentration of activity with respect to nitrogen. The enzyme was found to be stable in either homogenates or soluble fractions for at least 3 to 5 hours at 2°. After removing 0.6 ml of each spleen homogenate for adenosine deaminase assays and nitrogen analyses, remainder of each homogenate was centrifuged at 56,550 $\times$ g for 30 minutes. All supernatant samples were carefully decanted into chilled tubes and volumes recorded. For guanase assays, the cuvettes contained 0.025 or 0.05 ml of the soluble fractions, 1 ml of 0.1 M phosphate buffer, pH 7.4, water to 3 ml, and 0.42 μmole of guanine. Readings were taken at 2-minute

TABLE I. Effect of Whole-Body X-Irradiation on Specific Activity of Adenosine Deaminase of Rat Spleen Homogenates.

Exp.	Group	Avg specific activities* with ranges at varying days after irradiation 1	2	3	4	5	8
I	C†	.109‡ (.091-.120)	.096 (.084-.110)	.107 (.104-.108)		.115 (.107-.126)	.094 (.082-.100)
	X	.132 (.120-.143)	.138 (.112-.172)	.141 (.125-.169)		.112 (.105-.124)	.115 (.104-.133)
II	C	.114 (.093-.139)	.122 (.088-.153)	.114 (.102-.122)			
	X	.144 (.120-.178)	.116 (.082-.138)	.118 (.067-.172)			
III	C	.088 (.076-.105)	.110 (.101-.116)		.100 (.091-.109)		
	X	.103 (.093-.110)	.129 (.104-.142)		.118 (.100-.137)		

* μmoles adenosine deaminated/min./mg N. † C = control, X = irradiated.
‡ Each value represents avg of 4 animals.

intervals for 8 minutes. All assays for guanase and adenosine deaminase were carried out as soon as possible at 2 levels of tissue at a temperature of 25° with a thermostated Beckman model DU instrument. Activities were directly proportional to concentrations of tissue used, and reaction rates were linear during periods of observation. Specific activity of each enzyme was calculated by use of the molar extinction coefficients reported by Kalckar(5). Total activity was obtained by multiplying specific activity by total nitrogen. Nitrogen in homogenates and soluble fractions was determined by a micro-Kjeldahl procedure.

Results. For the 3 experiments, specific activities of adenosine deaminase are presented in Table I. Intervals studied after irradiation are listed in the table, for convenience, as days 1, 2, 3, 4, 5, and 8. Actually, these figures represent postirradiation periods of: 15, 39, 63, 87, 111, and 183 hours. In Exp. I, activities of irradiated rats were increased above control level by 21, 44, and 32% on the first, second, and third days, respectively, after irradiation. By day 5, activities had returned to normal level. In Exp. II, spleen adenosine deaminase activity was increased again on first day after exposure (+26%) but returned to control level on second and third days. In Exp. III, adenosine deaminase activity was uniformly 17% higher than that of controls on first, second, and fourth days after irradiation. While the results of each experiment were not identical, the trend towards a small increase in activity was consistent. In considering the results of Exp. II where no change in adenosine deaminase activity was found on days 2 and 3, the fact that the rats were much younger than those used in Exp. I and III should be taken into account. In Exp. I, employing combined control and irradiated spleen homogenates, no evidence was obtained for formation (or release) of a deaminase activator or destruction of a deaminase inhibitor. Table II summarizes results obtained for specific activities of guanase in spleens of normal and irradiated rats. On the first, second, and fourth days after irradiation, activity was increased by 34, 20, and 24% respectively.

Despite the general increase in specific activity of both enzymes, total organ adenosine deaminase and guanase activities decreased (Table III) due to considerable loss in spleen nitrogen (−35 to −50%) following irradiation. In general, spleens of X-rayed animals showed

TABLE II. Effect of Whole-Body X-Irradiation on Specific Activity of Guanase of Rat Spleen Supernatant Fractions.

Group	Avg specific activities* with ranges at varying days after irradiation 1	2	3
Control	.030† (.028-.032)	.037 (.031-.042)	.038 (.034-.040)
X-irradiated	.041 (.036-.045)	.044 (.042-.048)	.047 (.043-.050)

* μmoles guanine deaminated/min./mg N.
† Each value represents avg of 4 animals.

TABLE III. Effect of Whole-Body X-Irradiation on Total Activities of Adenosine Deaminase and Guanase of Rat Spleen.

Enzyme	Exp.	Group	Avg total activities* at varying days after irrad. 1	2	3	4	5
Adenosine deaminase	I	C†	4.33	4.04	4.98		2.92
		X	3.58	3.99	2.94		2.87
	II	C	1.63	1.62	1.62		
		X	1.13	1.14	.87		
	III	C	1.33	1.43		1.28	
		X	1.08	.96		.80	
Guanase	III	C	.187‡	.194		.183	
		X	.155	.109		.092	

* Values represent avg spleen total enzyme activity (μmoles adenosine or guanine deaminated /min./spleen). Each value represents avg of 4 animals.

† C = control, X = irradiated.

‡ Values represent activity of whole soluble fraction.

a 17 to 45% decrease in total adenosine deaminase activity between 1 and 4 days after irradiation. Total guanase activity present in the soluble fractions obtained from homogenates of X-irradiated rats averaged 17, 44, and 50% less than that found in corresponding fractions of control spleens during the 3 days studied. Total adenosine deaminase activity of control spleens in Exp. I was 2.5 to 3 times greater than that of control spleens in Exp. III. This discrepancy in total activity is due to the much larger spleens found in the rats used in the first experiment, despite the fact that the animals weighed about the same in Exp. I and III. The reason for the wide variation in organ weights is not known, but it is of interest that the specific activity of adenosine deaminase in control spleens (Table I) is independent of organ size. Hence, it would appear that the hypertrophied spleens studied in Exp. I did not affect activity of the enzyme.

Discussion. As in the case of another purine-metabolizing enzyme of spleen studied earlier, namely inosine phosphorylase(1), both adenosine deaminase and guanase have been shown in the present paper to undergo small increases in specific activity following 600 r of whole-body X-irradiation. On the other hand, total organ activity of each of the 3 enzymes was significantly decreased as compared to that of control animals. In agreement with Feinstein's(6) interpretation of increased specific activity of inosine phosphorylase, we recently(7) ascribed this and many other modest increases in spleen enzyme activity to an increased concentration of the enzymes with respect to nitrogen due to the marked nitrogen loss seen in spleens of irradiated animals. Based on the consideration of all combinations of changes in spleen enzyme activities that may be encountered, we presented a guide to interpretation of enzyme changes in spleen and other organs which undergo atrophy with a corresponding nitrogen loss after irradiation(7). According to this guide, spleen enzymes may be classified as follows: if specific activity is unchanged but total activity is decreased, the enzyme falls into case 1; if both specific and total activities decrease, the enzyme falls into case 2; if specific activity increases up to 100% but total activity decreases or remains the same, the enzyme falls into case 3a; and finally, if, along with a nitrogen loss of 50%, specific activity increases by more than 100% and total activity increases, the enzyme falls into case 3b. Under the experimental conditions employed, both adenosine deaminase and guanase may be classified in case 3a (Tables I to III). This means that increases in specific activity of these enzymes cannot be interpreted as activation of enzymes or as being due to enzyme synthesis but rather reflect an enrichment of enzymes with respect to nitrogen. It has been pointed out(7), based on a survey of results reported in the literature, that moderate increases in specific activities of spleen enzymes after whole-body irradiation may be more the rule than the exception. Or, it would appear more likely, for any spleen enzyme selected at random, that its

specific activity will be somewhat increased rather than decreased or unaffected. Such moderate increases in specific activity, unaccompanied by increases in total activity but usually associated with a loss of enzyme units, indicate that some enzyme has in fact been destroyed but rate of destruction has been exceeded by rate at which other nitrogen has been lost. These factors should be borne in mind in any comparison of response to irradiation of the same enzyme in a radiosensitive tissue and in a relatively radioresistant one.

Finally, it may be of interest that according to Smith and Low-Beer(8), 2 spleen enzymes involved in pyrimidine metabolism, uridine phosphorylase and cytidylic acid phosphatase, exhibited a slight increase in specific activity, and no increase in total activity (*i.e.*, case 3a) in rats exposed to 650 r, while a third enzyme, uridylic acid phosphatase, showed decreases in both specific and total activities (*i.e.*, case 2).

Summary. Adenosine deaminase and guanase activities were studied in homogenates and supernatant fluid fractions, respectively, prepared from spleens of control rats and animals given 600 r of whole-body X-irradiation. Specific activities of both enzymes were increased slightly in irradiated animals but total organ activities of both enzymes were decreased as compared to levels obtained in control rats. Interpretation of these and similar findings is discussed, and it is concluded that they do not represent direct or indirect enzyme activation or enzyme synthesis but rather reflect loss of nitrogen content and weight which are concomitants of splenic involution.

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Maintenance of Gonads of *Xenopus laevis* in Organ Cultures.* (26025)

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Some *in vivo* and *in vitro* effects of testosterone on gonads of larvae of *Rana catesbeiana* and *Xenopus laevis* have recently been reported(1). The present studies were made to determine whether gonads of *Xenopus,* placed in organ cultures at varying stages of development, would differentiate and respond to treatment with estrogen and chorionic gonadotropin in the same manner as *in vivo.*

Materials and methods. Larvae were made bacteriologically sterile(2) and cultures were set up as previously described(3). One part of glass distilled water in the medium was replaced by the hormones, estradiol (Progynon, Schering Corp.) or chorionic gonadotropin (Antuitrin-S, Parke, Davis and Co.). Developmental stages of larvae are numbered according to Nieuwkoop and Faber(4).

Results. Controls. Undifferentiated gonads from larvae of stages 49-50, attached to mesonephroi, showed no development in culture. In sectioned material no gonads nor germ cells could be positively identified. However, in gonads from larvae of developmental stage 52 and above, ovaries and testes could be distinguished. Some of these showed pro-

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TABLE I. Results of *In Vitro* Treatment of Gonads of *Xenopus* with Hormones.

Stage	Medium	Hormone	Days treated	Total animals	Males	Females	Undiff.	Undet.
49-55	Standard	None	5-56	49	15	18	4	12
51-60	"	Ch. gonado., 100 I.U.	11-59	36	11	10	0	15
55-65	"	Estradiol, 20 μg	14-56	44	17	15	0	12
56	"	" , 10 μg	22	16	5	4	0	7
Adult ♂	"	" , "	22	8	8	0	0	0

gressive development comparable to control animals *in vivo*. The sex ratio of gonads placed in culture was 1:1 as in intact control animals (Table I). In cultured gonads it was difficult definitely to determine the sex in every case since cells of the organs migrated to such an extent that the form of both cells and organs was modified.

Estradiol. Gonads from larvae in stage 55 were the youngest placed in cultures containing estradiol. Maintenance of cultured gonads on this medium was equally as good as in those kept in culture on standard medium without added hormone. Neither gonads of this stage nor those from older larvae or adults showed any modification of sex ratio.

Chorionic gonadotropin. The response of gonads to this hormonal substance was similar whether gonads were in culture or in intact animals. There was no evidence of sex transformation in either situation. In older male larvae the seminiferous tubules were more distended than in controls. There was hyperplasia of the rete apparatus in both ovaries and testes.

Discussion. Recent reports(1,5) give results following *in vitro* treatment of larval gonads of *Rana catesbeiana* with chorionic gonadotropin, estrogen, and androgen. In comparing these results with responses of *Xenopus* gonads it was found that chorionic gonadotropin caused hyperplasia of rete cells in both genera. No modification of gonads was observed in either group of larvae following estrogen administration. Androgen caused reversal of ovaries to testes in *Rana*, but in *Xenopus* there was only a retardation of development(1).

Gallien(6,7) and Chang and Witschi(8,9) found that testes were reversed to ovaries in *Xenopus* larvae treated with estrogens, but we found no similar change in gonads maintained in cultures with estradiol. The critical time in development when estradiol has its most pronounced effect corresponds to stages 48 through 55 when sex differentiation occurs according to Chang and Witschi(9). They state that at this time estradiol interferes with gene action, depressing the medullary inductor system and causing genetically male larvae to develop permanently into females. The fact that testes grown *in vitro* retained their male characteristics in the presence of estrogen may perhaps be due to the inability of gonads to be successfully maintained as typical organs prior to stages 58-60. Estradiol had little effect on the medulla of an already well-differentiated gonad *in vitro*.

Summary. Our study indicates that ovaries and testes, from larvae of *Xenopus laevis*, placed in organ cultures show progressive development, but gonads removed from larvae prior to or during time of sex differentiation do not differentiate *in vitro*. Older larval testes retain their normal male characteristics even on a medium containing estradiol. Chorionic gonadotropins stimulate rete apparatus of both ovaries and testes in organ cultures as well as in intact animals.

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Effect of Adrenaline, Noradrenaline and Chlorpromazine on Blood Pressure of Normal and Cold-Adapted Animals. (26026)

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In normal animals adrenaline, which is primarily secreted by the adrenal medulla, is essentially a metabolic activator as evidenced by its effect on oxygen consumption, adrenal ascorbic acid, etc.(1,2,3). On the other hand noradrenaline, which has little activity in that respect, is much more active on vasomotor control of blood pressure and is known as the physiological mediator of adrenergic vasomotor nerves(3,4). In animals exposed to cold for long periods of time, noradrenaline acquires a very strong calorigenic effect(5,6). However it is not known if adaptation to this stress will also change the vasomotor response to both adrenaline and noradrenaline. The present study reports the effect of these amines on blood pressure of cold-adapted rats and the effect of chlorpromazine on this response. We have shown previously that the hypothermic response to chlorpromazine is reduced in cold adapted animals(7).

Material and methods. Two groups of 10 male white rats, having a final weight of about 400 g, were used. The experimental group had been exposed to cold (10°C) for 6 months prior to test while the control group was kept for the same length of time at 25°C. The animals were anesthetized with nembutal (50 mg/kg i.p.) and heparinized. Blood pressure was measured by cannulation of the carotid and recorded on a kymograph by means of a double membrane liquid manometer. All drugs, which were given by the femoral vein, were contained in 0.2 cc volumes. Duration of all injections was 5 seconds. Both adrenaline and noradrenaline were assayed on control and experimental groups. The doses expressed in γ of the base varied between 0.07 and 10 per kg. Injections of adrenaline and noradrenaline were alternated in the same animals and time was allowed between injections for return of blood pressure to original levels. In 6 animals of each group chlorpromazine (2 mg/kg)* was injected after the initial response to adrenaline and noradrenaline had been studied. Once the constant hypotensive level following chlorpromazine injections was obtained, the response to both adrenaline and noradrenaline was again investigated.

Results. A. *Effect of adrenaline and noradrenaline on blood pressure of normal and cold-adapted rats.* The initial blood pressures of normal animals (108 $\pm$ 6) were not significantly different from those of cold-adapted animals (113 $\pm$ 5). Blood pressure increase following injections of adrenaline and noradrenaline in normal animals was directly proportional to dose and response to both substances was of the same magnitude (Table I). However, in cold-adapted rats, at doses of 0.5 and 0.2 γ/kg, response to noradrenaline was significantly greater than that to adrenaline. Similarly the response of cold-adapted rats to the 2 lower doses of noradrenaline was greater than that of normal rats. Fig. 1 illustrates these differences.

B. *Effect of chlorpromazine on blood pressure response of normal and cold-adapted rats to adrenaline and noradrenaline.* Normal and cold-adapted rats exhibited a similar hypotensive response to chlorpromazine. The quantity of chlorpromazine used (Table II) was proven completely effective in preventing the hypertensive response normally observed with adrenaline. However it was possible with a similar dose of noradrenaline to oppose to some extent the hypotensive response to chlorpromazine in both groups. Furthermore this effect of noradrenaline was significantly more pronounced in cold-adapted animals (Fig. 2).

Discussion. Adaptation to cold was known to increase metabolic activity of noradrenaline (5,6). In the present study we have shown that animals adapted to this stress are also more sensitive to the cardiovascular effect of noradrenaline. Furthermore this increased

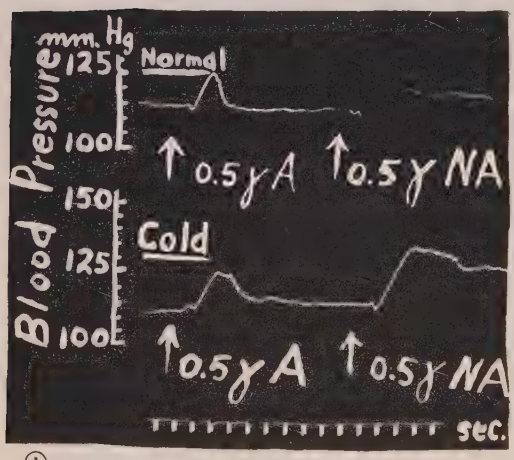
* Chlorpromazine was graciously supplied by Poulenc Ltd under their trade name Largactil.

TABLE I. Increase in Blood Pressure of Normal and Cold-Adapted Rats (10°C for 6 Months) Following Injections of Adrenaline and Noradrenaline.

Adrenaline dose (γ/kg)	Blood pressure increase (mm Hg) Normal	Cold	Noradrenaline dose (γ/kg)	Blood pressure increase (mm Hg) Normal	Cold
2	40 ± 6*	42 ± 5	2	36 ± 6	49 ± 4
1	27 ± 4	29 ± 4	1	30 ± 9	38 ± 2
.50	13 ± 3	14 ± 3	.50	14 ± 3	†*26 ± 3*
.25	5 ± 2	8 ± 3	.25	7 ± 3	†*25 ± 2*

* Stand. error.

† Significant at 1% level.



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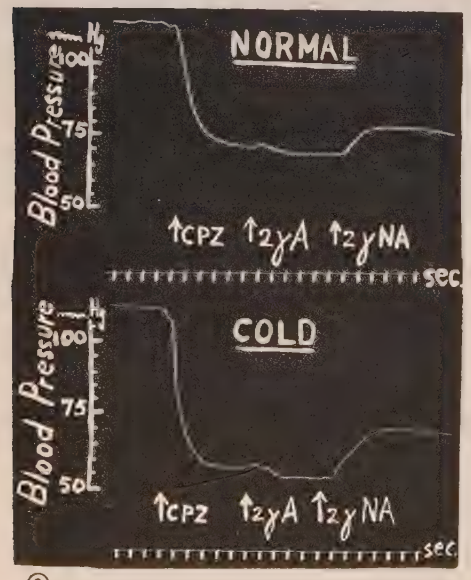

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FIG. 1. Blood pressure response of normal and cold-adapted (10°C for 6 mo) rats to adrenaline and noradrenaline i.v. injections.

FIG. 2. Effect of CP2 (chlorpromazine at 2 mg/kg) followed by adrenaline and noradrenaline in normal and cold-adapted (10°C for 6 mo) rats.

reactivity of cold-adapted animals to noradrenaline is observed only when smaller doses are given, *i.e.*, doses approaching physiological levels.

As pointed out by Von Euler(3) most of the adrenolytic agents and many antihistamines(8) antagonize much more the circulatory effects of adrenaline than of noradrenaline. This is also true, as we have shown, for chlorpromazine which is known to possess adrenolytic and antihistaminic action. Furthermore after adaptation to cold noradrenaline is more active than normally in opposing the hypotensive effect of chlorpromazine. Thus here also animals adapted to cold are more sensitive to noradrenaline than non-adapted animals.

This increased responsiveness of cold-adapted animals to noradrenaline may be related to recent results indicating that these adapted animals excreted much more noradrenaline than normal animals(9).

Summary. The blood pressure response of normal and cold-adapted rats to adrenaline, noradrenaline and chlorpromazine was studied. Adaptation to cold increased the re-

TABLE II. Change in Blood Pressure in Normal and Cold-Adapted Rats (10°C for 6 Months) Injected with Chlorpromazine Followed by Adrenaline and Noradrenaline.

Substance inj.	Blood pressure changes (mm Hg) Normal	Cold
Chlorpromazine (5 mg/kg)	−41 ± 8*	−53 ±11
Chlorpromazine + adrenaline‡ (2 γ/kg)	− 3 ± 1	− 1 ± 1
Chlorpromazine + noradrenaline‡ (2 γ/kg)	7 ± 2	† *18 ± 3*

* Stand. error.

† Significant at 1% level.

‡ Difference between (chlorpromazine) and (chlorpromazine + adrenaline) or noradrenaline.

sponsiveness to noradrenaline but not to adrenaline. The hypotensive response to chlorpromazine was the same in normal and cold-adapted rats and adrenaline did not oppose this effect in both groups. However noradrenaline, which normally slightly antagonized the hypotension of chlorpromazine, was much more effective in that respect when tested on cold-adapted animals. Cold adapted animals were known to be more sensitive to the metabolic effect of noradrenaline; the present study indicates that this increased responsiveness to noradrenaline also exists for its cardiovascular effect.

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Gonadotropic Antiserum Effects in Normal and Irradiated Female Mice.* (26027)

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Antisera to crude sheep pituitary extract with antigonadotropic properties inhibited development and differentiation of ovaries transplanted to the spleen of castrate mice and modified changes usually seen in mouse ovaries after irradiation(1,2). The effects of sera with pituitary antigonadotropic properties upon reproductive organs of normal animals have been differently recorded by others and experimental circumstances have varied (3-7). Therefore, it was deemed necessary to extend observations made on experimentally altered animals to normal young adults using the same materials and procedures. Particular attention was given to gonadotropic content of pituitary glands.

Materials and methods. Female mice of the MFl strain‡ 60-70 days of age at time of first injection were used. Two-tenths ml of antigonadotropic serum was injected subcutaneously daily 5 times a week for 6 weeks. Controls received normal rabbit serum, sheep heart antiserum, or saline; others were not injected. Some mice were x-rayed with injection starting the day after total body exposure to 175 r.§ Details of the method of preparation and assay of antiserum have been given (1,2,8). Briefly, the antiserum is developed by injection of sheep pituitary extract into rabbits until a gonadotropic antagonistic effect appears in the serum. The effect is demonstrable when pituitary extract (the antigen) and antigonadotropic serum (the antibody) are injected simultaneously, but separately, into immature female mice. Preparations comparable to those used in this study inhibit endogenous gonadotropins(9) and are aspecific(10). Thyrotropic activity is measurable,‖ but there appear to be negligible amounts of lactogenic activity. And, on the basis of short term assays, there is little adrenocorticotropic or growth hormone ac-

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† The author was assisted by Rosemarie Tuercke and Edith Reismann.

‡ Purchased from Manor Farms, Staatsburg, N. Y.

§ Physical factors have been given in detail in (2).

‖ Preliminary studies do not indicate that I^{131} uptake after injection of pituitary extract is altered.

TABLE I. Effect of Antigonadotropic Serum on Irradiated and Non-Irradiated Female Mice.

Treatment	No. animals	Body wt (g)	Ovarian wt (mg)	Uterine wt (mg)	Splenic wt (mg)	Thymic wt (mg)	"Wheel"* cells (%)
1. a. None	22	30.5	13.7	99.5	217.9	53.3	20
b. X-ray alone	10	29.0	7.4	84.2	187.9	56.0	
2. a. Saline	19	30.3	10.3	100.9	188.9	49.1	41
b. X-ray + saline	19	29.3	7.0	80.9	244.2	54.2	
3. a. Normal serum	20	30.6	9.2	96.0	315.2	53.1	54
b. X-ray + normal serum	21	30.9	5.9	74.1	308.1	53.2	
4. a. Sheep heart antiserum	9	31.4	10.5	76.6	318.2	43.5	50
b. X-ray + sheep heart antiserum	9	31.2	6.5	61.1	278.6	48.6	
5. a. Antigonadotropic serum	29	32.2	8.6	61.3	375.0	51.1	89
b. X-ray + antigonadotropic serum	27	33.0	3.1	40.1	359.0	55.2	

Probability (P)†	Body wt	Ovarian wt	Uterine wt	Splenic wt
5a *vs* all controls		.4	.001	
5a " normal serum	.05	.5	.02	.1
5a " saline	"	.2	.01	
5a " no treatment		.001	.001	
5b " all controls		.001	.001	
5b " normal serum	.05	"	"	.1
5b " saline	.001	"	"	
5b " no treatment		"	"	

* % ovaries with "wheel" cells indicative of gonadotropic deficiency.

† P values based on SD $= \sqrt{\frac{\Sigma d^2}{N-1}}$.

tivity(11). At autopsy, 6 weeks after irradiation on the third and fourth day after last injection, pituitary glands were removed and frozen until used for assay. Other organs were fixed in Bouin's solution and prepared for histological study by the paraffin method. Hematoxylin and eosin and periodic acid-Schiff and hematoxylin stains were used. Vaginal smears were taken by saline lavage daily 6 days each week during the injection period. The animals were housed in groups of 5 in cages measuring 5x6x12 inches and constantly supplied with water and a mixture of Rockland Farms Mouse Diet and Purina Chow pellets. Four series of 20 mice each were treated at different times between September and January. For assay, single pituitary glands were homogenized in 1.5 ml saline and injected into C57 Black females¶ 22-24 days of age. One-quarter ml of the solution was given twice daily for 3 days with autopsy following on the morning of the fifth day, when ovaries and uteri were weighed.

Results. Organ weight data are recorded in Table I. Ovaries and uteri of mice given antiserum weighed less than controls within irradiated or non-irradiated groups; they weighed most after saline was injected or when no injection was made. As a rule, the weights of ovaries and uteri from animals which had received either normal rabbit serum or sheep heart antiserum were intermediate. Ovaries and uteri of irradiated mice weighed less than those of non-irradiated mice. Differences between ovarian weights of controls after normal rabbit serum when compared with those of antihormone injected females were significant only in case of irradiation. Uterine weight differences, however, were significant throughout.

Essentially similar results were obtained by Rowlands(6) who concluded that persistent corpora lutea in mouse ovaries were probably responsible for maintaining ovarian weights after antihormone treatment. In this

¶ Purchased from Rockland Farms, New City, N. Y.

TABLE II. Effect of Antigonadotropic Serum and Irradiation on Pituitary Gland Gonadotropic Potency.

Material inj. into assay mice	Treatment of donor mice	Assay mice: No. animals	Body wt (g)	Ovarian wt (mg)	Uterine wt (mg)
Nothing (control)	——	34	11.3	3.0	6.0
Sheep pituitary extract*	——	6	13.6	5.9	22.6
Pituitary from donor mice	Castration	28	12.9	3.8	14.9
	No treatment	17	11.3	2.7	7.4
	X-ray + no treatment	8	12.3	3.4	20.8
	Saline	15	11.5	2.7	8.7
	X-ray + saline	17	11.5	4.3	18.6
	Sheep heart antiserum	8	10.8	3.0	8.2
	X-ray + sheep heart antiserum	9	10.2	4.1	20.9
	Normal serum	19	10.9	3.2	8.4
	X-ray + normal serum	21	11.9	4.0	16.9
	Antigonadotropic serum	28	11.9	3.6	15.0
	X-ray + antigonadotropic serum	24	10.6	3.8	13.3

* Equivalent of 25 mg of acetone dried pituitary glands.

study, 11% (4/37) of the antihormone treated non-irradiated ovaries had corpora lutea, whereas 52% (13/25) of ovaries of mice treated with normal serum had corpora lutea. They were present in 90% (27/30) of uninjected mice. Apparently normal follicles with large antra were present in about 80% of ovaries of controls and in 64% of ovaries from mice given antihormone but not irradiated. After irradiation less than 35% of ovaries, irrespective of treatment, had an occasional abnormally small corpus luteum or Graffian follicle.

Cytological differences were evident in ovarian interstitial tissue. Cells with coarsely granular hyperchromatic nuclei were more frequently encountered in ovaries from mice given antihormone (Table I). In addition, the number of these cells per section and intensity of development of hyperchromasia was greater after injection of antihormone. These cells resembled the "wheel" cells which, according to Selye *et al.*(12), appear shortly after hypophysectomy. Essentially similar results were suggested after examination of a few irradiated mouse ovaries confirming earlier studies(2).

On the basis of criteria established by Hooker and Forbes(13) for the mouse uterus, 53% (10/19) of the uteri from non-irradiated groups given antihormone showed slight or no evidence of estrogenic and/or progesterone-like effects. About 20% (4/14) of the uteri in normal serum-treated groups were similarly unstimulated. Uteri of irradiated mice presented much the same response.

Irradiation as well as injection of gonadotropic antiserum increased the gonadotropic potency of pituitary glands to the same degree as in glands from castrate mice. Other injected materials had no effect (Table II). Mandl and Zuckermann(14) reported increased gonadotropic activity within the pituitary glands of irradiated rats, and Levine and Witschi(15) demonstrated increased circulation of gonadotropic hormones after rats were exposed to x-rays. A similar effect on circulating hormones in parabiotic mice after irradiation was shown by Vermande-Van Eck and Chang(16). Meyer *et al.*(18) showed that injection of an antigonadotropic serum under conditions somewhat comparable to those used in this study, increased the gonadotropic content of pituitary glands of rats. The present study indicates that, as in rats, there is enhanced storage of gonadotropic hormones in pituitary glands of mice after antihormone and after irradiation. It would appear that the well known disparity in ultimate outcome of effects of irradiation upon ovaries in these forms does not lie with initial response of the pituitary gland to altered ovarian function.

Typically vaginal cycling continued

throughout the period of observation regardless of treatment. However, the cycles of mice given antihormone were initiated consistently at a fixed time after the fifth injection. It was concluded that there was a release of increased amounts of gonadotropic hormone at these intervals which was responsible for initiation of a new cycle. There were no important differences in length or frequency of vaginal cycles among experimental groups. However, occurrence of vaginal smears with a preponderance of cornified cells was less frequent after antihormone.

Grossly the ovaries of assay mice which received one pituitary gland from donors irradiated, castrated, or treated with antihormone had much the same appearance. The follicles were somewhat cloudy and there was an occasional corpus luteum, whereas follicles of controls were clear, and there were no corpora lutea. Uteri of all assay mice were opaque and had no intraluminal fluid. Those which were stimulated by injection of pituitary glands from mice given antihormone, irradiated, or castrated had heightened epithelium and a slight submucosal edema. The stromal nuclei were fusiform with dense chromatin. Results with the method used here suggest that a single gland of a mouse irradiated with 175 r or given antihormone has sufficient FSH and LH to stimulate release of estrogen from the ovary of the recipient, but that there is slight but insufficient luteotropin present for fully developed luteinization and progesterone formation. In the immature rat Maddock *et al.*(17) concluded that thick-walled, opaque uteri devoid of fluid indicated the presence of both estrogen and progesterone.

Particular attention was given to selection of control materials, since the influence of exogenous factors upon gonadotropic storage by the pituitary gland is well known. For example inanition appears to increase gonadotropic content per milligram of gland(19, 20). Protein deficiency may or may not increase gonadotropic levels of pituitary glands, but in any event it does not prevent the expected increase in gonadotropic activity of the pituitary gland after gonadectomy(21,22). Vit. A deficiency has been shown to elevate gonadotropic potency of the pituitary gland (23), and some reports indicate a similar effect when Vit. E is deficient(24). Herlant (25) reported hypertrophy and hyperplasia of anterior lobe basophiles in rats induced by a wide variety of stressing agents. Nonspecific stress of sufficient magnitude to bring about atrophy of gonads and accessories frequently, if not invariably, induces thymic involution, adrenal hypertrophy, and body weight loss(26). It has been estimated that for each gram of body weight loss there is a 10% decrease in number of estrous cycles (27). In the present study mice which had received antihormone were slightly heavier than controls (Table I), and number of cycles could not be shown to differ among injected groups. Changes in thymic weights were not marked (Table I). Kidneys and thyroids of about one-half of the animals were studied histologically and appeared to be essentially normal. Adrenal glands did not reveal differences among variously treated animals, and measurement of width of the cortex was not pursued after preliminary trials proved unprofitable. Thus it may be noted that gonadotropic content of pituitary glands was not elevated in mice receiving heterologous normal serum or sheep heart antiserum as it was after antigonadotropic serum. There was no evidence of nonspecific stress in control animals.

Summary. Normal and irradiated female mice were given gonadotropic antiserum. Controls received normal rabbit serum, sheep heart antiserum, or normal saline solution. After antihormone serum treatment ovarian and uterine weights were below those of controls. Ovaries of mice after injection of antihormone serum had fewer corpora lutea than did controls, and interstitial tissue gave evidence of diminished gonadotropic stimulation as indicated by increased frequency of cells indicative of gonadotropic deficiency. Uteri lacking histological evidence of stimulation were found most frequently in animals given antihormone serum. Ovaries and uteri of irradiated animals responded in essentially similar manner to treatment, but the response was more extreme. Irradiation and gonadotropic antiserum increased gonadotropic po-

tency of pituitary glands; neither normal rabbit serum nor sheep heart antiserum had any effect. A pattern of vaginal cycling was observed after antihormone treatment which was not apparent in cycles of controls. No important differences were found in thymus weight or in adrenal or thyroid histology.

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Effects of Psychotropic Drugs on Limbic System of Cat.* (26028)

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There has been increasing evidence that the limbic system (rhinencephalon) is involved in emotional responses(1). This suggests that drugs which alter mood might produce changes in this part of the brain. The present paper describes effects on the septum, amygdala and hippocampus of the cat of 2 psychodepressant drugs (chlordiazepoxide† and meprobamate) and 2 psychostimulant agents (iproniazid and imipramine).

Methods. Thirty cats were prepared under ether anesthesia. The trachea was cannulated for artificial respiration and the left femoral vein for drug injection. Bipolar concentric electrodes were inserted with the aid of a Baltimore stereotaxic instrument in anterior and posterior portions of septum, amygdala and hippocampus. Recording electrodes were placed on frontal and occipital areas of cortex. All wound edges were infiltrated with procaine. The ether was withdrawn and the animals immobilized with decamethonium (C_{10}). Stimulation was carried out with a Grass S-4 stimulator, using the following parameters: frequency, 40 per sec.; pulse

* A brief report was presented to Am. Soc. Pharmacol. and Exp. Therap., Seattle, Wash., Aug. 21, 1960.

† Librium® formerly referred to as methaminodiazepoxide.

TABLE I. Statistically Significant Drug Effects. Figures show mean ± stand. error of mean.

Site of stimulation	Drug	Dose, mg/kg i.v.	No. of exp.	Duration of after-discharge, sec. Pre-drug	Post-drug*	Difference	Statistical significance‡
Septum	Saline		4	31 ± 8.7	66 ± 17	+ 35 ± 11	
	Chlordiazepoxide	10	3	39 ± 11	27 ± 7.9	− 12 ± 12	<.02
	"	20	4	42 ± 8.3	19 ± 3.5	− 23 ± 10	<.01
	Imipramine	10	3	33 ± 14	1.7± .88	− 31 ± 14	"
	Meprobamate	20	4	39 ± 27	11 ± 6.1	− 28 ± 31	<.05
Amygdala	Saline		3	20 ± 12	21 ± 6.0	+ 1.3± 8.5	
	Iproniazid	5†	3	8 ± 2.9	64 ± 17	+ 56 ± 17	"
Hippocampus	Saline		4	23 ± 7.9	46 ± 11	+ 23 ± 9.2	
	Chlordiazepoxide	10	3	38 ± 7.5	23 ± 13	− 15 ± 8.5	"
				Amplitude of after-discharge, μV			
Amygdala	Saline		4	1300 ±436	1297 ±435	− 2.5± 2.5	
	Chlordiazepoxide	10	4	1060 ±410	310 ±110	−750 ±240	<.02
	"	20	4	" "	210 ± 60	−850 ±370	"

* Maximum effect.
† Pre-treated for 5 days with 5 mg/kg i.p. "Pre-drug" value immediately before inj. of 5 mg/kg i.v.
‡ "p" values for drug *vs* saline figures in "Difference" column.

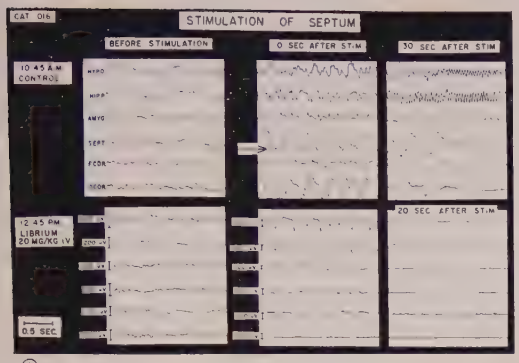
duration, 2 msec; intensity, 8 volts; duration of stimulation, 5 sec.; interval between stimulations, 5 min. Subcortical centers were stimulated in the following sequence: anterior septum, amygdala, hippocampus; posterior septum, amygdala, hippocampus. Recordings were made on a Grass III-C electroencephalograph. Only one drug was administered to each animal. Iproniazid was tested at 5 mg/kg i.v. in cats pre-treated for 5 days with 5 mg/kg i.p. The other drugs were tested in animals without pre-treatment; a dose of 10 mg/kg i.v. was followed 1 hr later by 20 mg/kg. To minimize cardiovascular effects, drugs were injected over a 10 min period at the rate of 1 ml/min. Significance of results was determined by "t" test. Electrode locations were confirmed histologically.

Results. (Table I): *Saline controls*: Stimulation of limbic centers produced after-discharges of high amplitude (1000 μV or more) with frequency of 3-21 per sec. These after-discharges spread throughout the limbic leads; they were usually marked by abrupt termination. Duration of discharge following successive stimulations remained relatively constant in the amygdala, but progressively increased in septum and hippocampus.

Chlordiazepoxide showed the following statistically significant effects at 10 mg/kg i.v.: decreased duration of after-discharge in septum and hippocampus; decreased amplitude of discharge in amygdala. *Meprobamate,* 20 mg/kg, shortened duration of discharge in septum. *Imipramine* showed the same effect at 10 mg/kg. *Iproniazid* had no effect in cats without pre-treatment; following administration of 5 mg/kg i.p. for 5 days, 5 mg/kg i.v. significantly increased the duration of discharge of the amygdala.

Discussion. Delgado(*2*) observed that stimulation of the amygdala in unanesthetized monkeys "increased aggressiveness and fighting." The increased activity of the amygdala which we observed with iproniazid might be involved in the psychostimulant effect of this drug. Delgado also observed that stimulation of the septum produced "loss of leadership." If the septum has a restraining influence on behavior, the depression of this area which we observed with imipramine might produce psychostimulant effects. However, depression of the septum could hardly produce both psychostimulant effects with imipramine and psychodepressant effects with meprobamate.

Kletzkin and Berger(3) studied the effects of meprobamate on the limbic system of the cat with a technic differing only slightly from ours (immobilization with succinylcholine, more frequent stimulation of 1 or 2 areas). They observed reduced duration of after-discharge in hippocampus and amygdala at 20 mg/kg i.v. If stimulation of the amygdala



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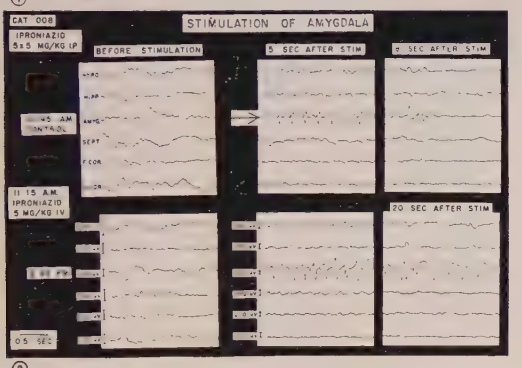

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FIG. 1. Chlordiazepoxide, 20 mg/kg i.v., decreases duration of discharge in septum. Upper row is before, lower row 30 min. after, drug inj. First panel in each row is before, second and third panels after, electrical stimulation of area indicated by arrow. Note reduced amplification in second and third panels. EEG leads are hypothalamus, hippocampus, amygdala, septum, frontal and occipital cortex. Paper speed 6 cm/sec.

FIG. 2. Iproniazid, 5 mg/kg i.v. in cat pretreated for 5 days with 5 mg/kg i.p., increases duration of discharge in amygdala. Arrangement as in Fig. 1.

increases aggressiveness, reduced activity of this area might have a psychodepressant effect.

We found that chlordiazepoxide at 10 mg/kg i.v. had depressant effects on septum, amygdala and hippocampus. In other tests, this compound was found to be more potent than meprobamate in depressing the irritability of rats with septal lesions, and in reducing the aggressiveness of vicious monkeys (4). In the clinic, this agent "reduces anxiety and agitation but does not cloud consciousness or impair intellectual function"(5). Perhaps these psychodepressant effects are correlated with depression of the amygdala; much more experimentation is needed before any such relationship can be established.

Summary. Four psychotropic drugs had the following statistically significant effects on the limbic system of the cat: iproniazid increased duration of after-discharge of amygdala; imipramine and meprobamate decreased duration of discharge of septum; chlordiazepoxide decreased duration of discharge of septum and hippocampus, and decreased amplitude of discharge of amygdala.

We wish to thank Shirley Yerzy for the histology and Kay Eisenhuth for statistical analysis.

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Effects of Selective Ultraviolet Irradiation of Cytoplasm of Living Cells.* (26029)

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The authors have reported some of the manifestations of ultraviolet irradiation damage in living cells(1,2). The effects reported concerned the nature of the response of the whole cell to ultraviolet irradiation, rather

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than effects of ultraviolet irradiation of selected portions of living cells. The purpose of this report will be to extend previous observations by recording additional types of ultraviolet induced irradiation damage in living cells, and to record some observations on response of the cytoplasm alone to ultraviolet irradiation.

Methods. We have previously reported the technic of ultraviolet flying spot television microscopy and time-lapse cinematography (3,4, 5). Recently the authors have recorded improvements in this technic which permit use of double flying spot illumination (6). In the experiments reported here the principle of double flying spot illumination was employed to illuminate the nucleus of the cell with light of a peak wave length of 4500 Å and to illuminate the cytoplasm of the same cell with ultraviolet light with a peak wave length of 2600 Å. Control cells in the same microscopic field were illuminated solely with ultraviolet light. The ultraviolet emitting scanner tube and the visible light emitting scanner tube—which in this case acted as the sources of illumination — were permitted to operate continuously at a scan rate of 4 frames per second. Every 7 seconds the monitor tube was selectively blanked so that a new 16 mm film frame could be pulled into position. Each 16 mm film frame stored the image information from 28 scanned frames of the monitor tube. By storing the monitor tube information from multiple scan frames on one 16 mm film frame the signal to noise ratio was greatly increased. During the experiments the equipment was periodically altered so that a complete ultraviolet absorption image of all cells was obtained. This ultraviolet absorption image was obtained and recorded on the film for several minutes at selected intervals and again for 30 minutes at end of experiment. Three types of cells were used in this experiment; HeLa cells, Chang Liver cells, and mouse fibroblasts. All of the cells gave identical results. All of the cells were carried in stock cultures in Eagle's media enriched with horse serum and for purposes of the experiment they were transferred to Rose chambers with quartz coverslips. During the course of the experiment only, the chambers were supplied with Eagle's media without horse serum. Horse serum was removed from the media during experiment because its absorption of ultraviolet light at these wave lengths obscured the nature of the absorption image of the cells. In all, these results represent data taken from 5,000 feet of 16 mm black and white film. A total of 1,000 cells was studied. Fifty of these were subjected exclusively to cytoplasmic ultraviolet irradiation while the remaining 950 cells acted as controls and were subjected to total cell ultraviolet irradiation.

Results. Ultraviolet irradiation applied to the whole living cell will result in a variety of damage patterns depending on dose and rate of dosage. The most acute pattern of this response is a sudden collapse of the cell from its normal spread-out attachment to the coverslip into a round ultraviolet opaque ball without attachment to the coverslip. This sudden collapse occurs at the end of 1 hour of scanning at a scanner tube beam current of 50 microamps and is entirely unheralded by a change in absorption of the cell or by a change in its motion. With less intense doses of irradiation given by a scanner tube beam current of 20 microamps several types of damage appear. These may be divided into cytoplasmic components and a nuclear component. The most subtle form of cytoplasmic damage consists of a gelling of the cytoplasm. This has 2 effects on cytoplasmic motion. One effect is to increasingly restrict, to the point of cessation, the motion of lipoprotein droplets of the cytoplasm. The second effect is completely to inhibit pinocytosis so that all movement of the cytoplasmic membrane is lost. These effects take place on the average after 2 hours of scanning. During the next several hours other cytoplasmic effects follow, consisting of several varieties of bubble formation. One type of bubble is a large nonabsorbing bubble forming on/or from the cytoplasmic membrane. These bubbles are apparently formed by syneresis. A second type of bubble differs from the first in that the bubbles in this instance are filled with cytoplasm and contents of these bubbles show ultraviolet absorption comparable to the remaining cytoplasm. Still a third type of bub-

ble which may follow development of the first two is the intracytoplasmic bubble. This type of bubble is usually multiple and is always transparent to ultraviolet light. All 3 types of bubbles have been observed to rupture and release their contents into the environs. In the end the cytoplasm loses virtually all of its absorption and it decreases markedly in area. During the time interval required for production of these cytoplasmic manifestations of damage the nucleus shows marked evidence of damage. In the undamaged cell the nuclear membrane is not visible as an ultraviolet absorbing structure; the nuclear sap shows slight ultraviolet absorption on the order of 10% of absorption of the cytoplasm adjacent to the nucleus, while the nucleolus shows intense absorption. As cellular damage due to ultraviolet irradiation progresses, a condensation of ultraviolet absorbing material takes place at the nuclear membrane. The nucleus progressively diminishes in size to approximately one-half its original state and the nuclear membrane appears to become more and more prominent. As the nucleus decreases in size, the increasingly prominent nuclear membrane becomes wrinkled and infolded. Together with these changes the nucleus displays a general increase in absorption, roughly paralleling the progressive decrease in cytoplasmic absorption. There is, however, no proof that the two are related other than in time. The increase in nuclear absorption may be as much as 4-fold. During these processes of cellular damage the nucleoli do not change size, shape or absorption.

When the experiment is arranged so that cytoplasm alone is irradiated by ultraviolet, the following results were noted. The cytoplasm of the cell with the protected nucleus shows the same responses to ultraviolet irradiation as does the cytoplasm of control cells in the field. These are the responses detailed in the preceding section, and they occur at the same time in cytoplasm of control cells in the field of irradiation as in the cell with the nucleus illuminated with visible light. Nuclear responses in protected cell and in control irradiated cells are quite different. Nuclei of the totally irradiated control cells show the typical nuclear responses to irradiation detailed in the preceding section. Nuclei of the protected cells do not show any of these responses. Their only change during experiments is a change in shape which accompanies the changing shape of the cell as the cytoplasm bubbles and finally retracts to give the cell a rounded shape. Nuclear absorption of ultraviolet light at beginning, middle and end of the experiment remains the same as an unirradiated cell. The nuclear membrane never becomes an ultraviolet absorbing structure even in the face of the most severe cytoplasmic damage. Nucleoli in the protected nuclei remain unchanged in number, size, shape and ultraviolet absorption.

Discussion. Previous experiments in the literature dealing with the role of the nucleus in ultraviolet irradiation damage have been carried out differently from those reported here. These studies have involved single relatively large doses of irradiation to intact amoeba, or to enucleated amoeba, or to separated amoeba nuclei. The latter may be suitably recombined with cytoplasm to study the effects of transplanted irradiated or unirradiated nuclei, grafted into irradiated or unirradiated cytoplasm. Under these circumstances the unirradiated nucleus grafted into irradiated cytoplasm produces an increase in longevity as compared with irradiation of the intact organism(7,8,9,10,11). The experiment reported here differs so markedly from the above experiment that no comparison is possible. One of the major areas of variance is that in the experiment reported here the cytoplasm was continuously irradiated until damage appeared. A second difficulty rests in the fact that no existing technic may be applied to determine dose of irradiation given to the specimen by the flying spot microscope. Hence it is not possible to compare irradiation dosimetry.

It would appear from these experiments that continuous ultraviolet irradiation of the cytoplasm of the intact cell produces the same cytoplasmic effects as does continuous irradiation of the entire cell. In these experiments nuclear evidence of ultraviolet induced irradiation damage did not occur.

Conclusions. (1) Nuclear protection from

ultraviolet irradiation during continuous ultraviolet irradiation of cytoplasm confers no degree of protection on cytoplasm or on longevity of the cell. (2) Nuclear protection from ultraviolet irradiation during continuous ultraviolet irradiation of cytoplasm prevents nuclear manifestations of ultraviolet induced nuclear damage.

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Constancy of Gastric Acidity with Variable Secretory Rates Induced by Insulin or Glucagon, with Histamine.* (26030)

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There is now considerable evidence to controvert the old concept concerning gastric acid secretion according to which concentration of acid in the primary secretion, as it arises from the parietal cell, varies with volume rate of flow(1). In the experience of our group, it has been found that the acidity of pouch juice may remain constant at high levels (*e.g.*, in the neighborhood of 150 meq/l) for significantly long periods of time and under a variety of experimental conditions. This was first observed in dogs with Pavlov pouches, following a single subcutaneous injection of histamine of very high dosage; in these experiments, acidity maintained a plateau level for a long time, during which rate of secretion rose and then fell to a level of about 10% of its maximum(2). Similar evidence has been adduced from secretion induced by low dosages of histamine and by ingestion of food, when the juice was collected in such a way as to minimize the concomitant secretion of mucus(2); also during the diurnal hyper-secretory activity manifested by a bitch during lactation(3). Again, in experiments with Heidenhain pouch dogs in which both acidity and volume-rate were maintained at a fair degree of constancy, by repeated injections of histamine at short intervals (0.025 mg base every 10 minutes) for many hours, the superimposition of a single subcutaneous injection of atropine (0.4 mg/kg) about the middle of a 4-6 hour run, in order to inhibit the secretory response to the continuing administration of histamine, resulted in a drastic reduction in volume-rate without any marked change in acidity(4).

At no time, however, has it been reported that such essential lack of correlation between acidity and volume-rate obtains with purely vagal or vagomimetic stimuli, or even histamine at low dosage under ordinary collection conditions, but this may be ascribed to the fact that such stimuli evoke a gastric juice which is relatively high in organic matter—enzymes and mucus—and therefore relatively low in acidity. As a result of variations in these organic constituents, constancy of acidity is practically never attained, and time

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curves for HCl concentration and volume-rate manifest the usual parallelism. However, if one compares the acidities in such experiments at 2 or more times when the rates are identical, the lack of correlation is immediately evident. Thus, in histamine or food experiments with dogs, in which there was a manifest parallelism of the time curves for acidity and volume-rate, the data left no doubt that at 2 or more times during the experiment when volume-rate was the same (*e.g.*, near beginning and end of the secretory response), the acidities were so markedly different as to exclude any precise quantitative relation between the two variables(5). This same phenomenon can be seen from the data of an experiment on man by Olson and Necheles(6), in which the authors reported the usual stimulatory effect of an adequate insulin-induced hypoglycemia, along with a preceding inhibitory action of unknown mechanism. From the graphs in Fig. 1, it is evident that when a horizontal line, R-S, is drawn to transect the rate curve at 3 different positions or times, corresponding total acidity values, indicated by the verticals, are 53, 61 and 76 meq/l respectively. None of the experiments of these authors revealed a constancy of acidity like those with which we are dealing in the present report.

We recently completed a study of the inhibitory effect of insulin on histamine-stimulated canine gastric secretion in fully vagal pouches of the Hollander-Jemerin type(7) as well as in completely vagotomized (Heidenhain) pouches. The histamine was administered in repeated 10-minute subcutaneous doses of 0.1 mg of base for a total period of at least 4 hours. Some time after volume-rate of secretion had reached its maximum and leveled off (about 2 hours after start of experiment), a single dose of insulin (0.75 U/kg) was injected intravenously. This was followed promptly by a marked drop in secretory rate, similar to that reported by Olson and Necheles. In the case of the illustrative experiment shown in Fig. 2, volume-rate rose progressively during the first 75 minutes of repeated histamine injection, after which it remained constant for a full hour. Insulin injection at this point was followed by a rise in rate for a single 15 minute sample, and then a rather steady fall for the remainder of the experiment—from a peak of 6 ml/15 minutes to a low of 2.0 ml/15 minutes. In spite of this 3-fold variation in volume-rate, the acidity remained at a plateau for 3 hours, varying only within the limits of 138 and 151 meq/l—a range of ± 4.5% about its mean. Such prolonged constancy of acidity was observed

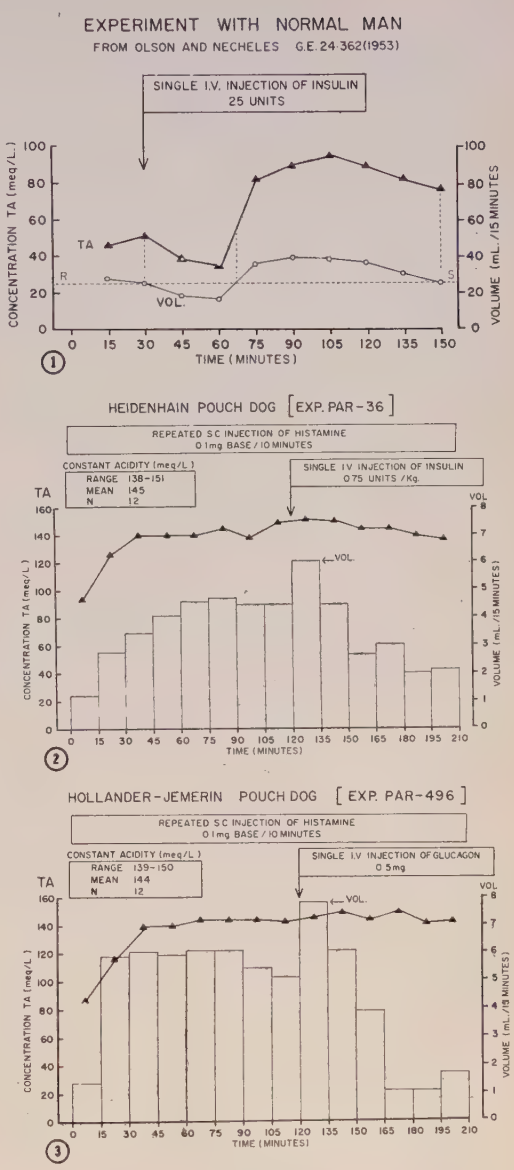

FIG. 1-3.

in 11 experiments in which the insulin-induced decrease in secretory rate persisted 90 minutes or more.

The same phenomenon has been observed in several similar experiments in which the secretion induced by repeated 10-minute injections of histamine was inhibited by a single intravenous injection of glucagon, Lilly‡ (0.5 mg/kg) in dogs with vagal pouches of our own variety.§ In the illustrative experiment presented in Fig. 3, the total acidity curve remains constant in the interval 139-150 meq/l (a range of ± 3.8% around the mean) for 3 hours, during which volume-rate varied from 7.8 to 1.1 ml/15 minutes.

Conclusion. We have now extended the variety of evidence regarding the constancy of gastric acidity—in refutation of the old concept of a curvilinear correlation between it and volume-rate of secretion—to include 2 additional types of stimulation-inhibition process. With these new findings, the list of experimental conditions for which this has been observed in pouch dogs now includes the following: (1) A single subcutaneous injection of histamine, at both high and low dosage levels, in Pavlov pouches. (2) Ingestion of food, which may be presumed to involve vagal as well as humoral stimulation. (3) Gastric hypersecretion during lactation. (4) Inhibition, by a single subcutaneous injection of atropine, of gastric secretion induced by small doses of histamine injected subcutaneously every 10 minutes, in Heidenhain pouches. (5) Stimulation and inhibition of secretion by a single intravenous injection of insulin, superimposed on the response to repeated 10-minute injections of histamine in Hollander-Jemerin pouches. (6) Similar inhibition of histamine-induced secretion by a single intravenous injection of glucagon, on these fully vagal pouches. In spite of this and other recent additions to the mass of evidence supporting the two-component hypothesis to explain observed variations in gastric acidity, it must be remembered that there exist several kinds of observations which are seemingly in conflict with it; experimental efforts to clarify these discrepancies between theory and fact are being continued.

Summary. This report deals with the absence of correlation of acidity and volume-rate of flow of gastric secretion from fully vagal (Hollander-Jemerin) and completely vagotomized (Heidenhain) stomach pouches. Variations in rate of secretion were induced by the inhibitory action of a single intravenous dose of insulin (both types of pouch) or of glucagon (vagal pouch only) on flow of juice induced by stimulation with histamine in repeated 10-minute subcutaneous doses. In both types of experiment it was possible to demonstrate a good degree of constancy of acidity in spite of considerable variations in volume-rate. This brings to 6 the number of experimental situations in which such constancy has been observed.

‡ The authors wish to express their thanks to Dr. Tsung-Min Lin of the Lilly Research Laboratories, for the glucagon used in these experiments.

§ Attention is called to our observation that completely vagotomized (Heidenhain) pouches failed to manifest such inhibition of histamine-induced secretion. This will be discussed in a subsequent report.

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On the "J" Classification of Rabbits and Production of Anti-J in "J-Negative" Rabbits. (26031)

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One of the chief problems in blood typing the J system of cattle is that of obtaining J antibodies which will lyse the red cells of all J-positive cattle to the exclusion of those bloods that are truly J-negative. The reason for the difficulty is that there are many genetic intergrades of J-positive bloods ranging from those in which the "J-coated" red cells have relatively little affinity for J antibody, and may not be lysed even by the best J reagents available, to those which have marked affinity for and are readily lysed by anti-J. Consequently, it has often been necessary to resort to inhibition tests for soluble J substance in blood plasma to distinguish the "lowest" intergrades of J-positive bloods from those that are truly J-negative. Until recently, the only source of anti-J has been the normal serum of J-negative cattle, but it is an unusual normal serum, if one exists, which will promote lysis of all J-positive cattle bloods. Various investigators(1,2,3,4,5) have attempted to enhance the titers and broaden the specificity of J isolysins by isoimmunization and some of these investigators have also attempted to produce anti-J by heteroimmunization. Although an immune response to cattle J substance has been claimed(2), most workers are convinced that there is little or no effect(1,3,4,5). It is now found that "J-negative" rabbits immunized with human red cells of type A regularly produce antibodies which cross react rather strongly with J-positive cattle bloods, and some of the J reagents obtained in this manner are, in the experience of the authors, equal to or superior to the best J reagents obtained from J-negative cattle. This report is concerned with production of such antibodies and with the "J" classification of rabbits.

Materials and methods. Mature, commercial rabbits of both sexes and of different breeds, primarily New Zealand Whites and Californians, were used. Prior to the immunizations, a sample of normal serum was collected from each rabbit and these samples were tested for their ability to inhibit lysis of J-positive cattle blood by anti-J, according to the following procedure. Samples of rabbit normal serum (inhibitant) were heat-inactivated for 1 hr at 56°C. To each of a series of 5 tubes (10 x 75 mm) was added 1 drop (app. 0.05 ml) of a rabbit's serum, diluted 1:1 in the first tube, 1:2 in the second, 1:4 in the third, 1:8 in the fourth and 1:16 in the fifth. To the one drop of diluted inhibitant in each of the 5 tubes was then added 2 drops of anti-J in a dilution sufficient to promote nearly complete hemolysis of J-positive red cells within 1½ hr, as judged by the positive control. (In the present study, we used our C86 normal serum diluted 1:50.) The tubes were then shaken and, after an interval of 10 minutes, 1 drop of a washed, 2.5% suspension of red cells from a J-positive donor was added to each tube. The tubes were again shaken and, after another interval of 10 min., 1 drop of freshly thawed, undiluted rabbit complement (a pool of fresh-frozen serum from 40 to 60 selected rabbits) was added to each tube. The tubes were again shaken and readings of hemolysis were recorded at 1½ hr and again after another 1½ hr. Controls consisted of a positive control (J antibody + red cells + 1 drop of saline in place of inhibitant), a complement control (saline in place of antibody and inhibitant) and a saline control. 0.91% NaCl solution was used as saline. All tests were performed at room temperature ranging from 23.5°C to 28.5°C.

The direct blood-typing tests were performed in much the same manner except that no inhibitant was used and that guinea pig complement diluted 1:15 was substituted for rabbit complement in the tests employing rabbit antisera or J reagents derived from rabbit antisera. In fact, the substitution of guinea pig complement for rabbit complement, which has heretofore been considered the comple-

ment of choice with all cattle blood-typing reagents, was the most crucial step in demonstrating the cross reactions of rabbit anti-human A with cattle J. The particular cross reactive lysins in such antisera are poorly activated with rabbit complement in contrast with guinea pig complement.

For the immunizations, each rabbit received a series of 6 intravenous injections, 2 per week, of 0.5 ml of a 50% suspension of red cells per injection. Antisera were collected and frozen 6 days after the last injection.

Six of 21 rabbits which were immunized with human red cells of type A received a series of 3 booster injections on alternate days beginning 54 days following collection of the antisera of the primary series, and these antisera of the secondary series (coded #2 in Table I) were collected 5 days after the third booster injection.

Results. Sera of 110 rabbits were examined for their ability to inhibit the lytic reactions of J antibody with J-positive cattle red cells. Serum of 54 of the rabbits showed no inhibition and were accordingly classified as "J-negative." Serum of each of the remaining 56 rabbits, classified as "J-positive," showed complete inhibition in the 1:1, 1:2 and 1:4 dilutions and most showed partial to complete inhibition in the 1:8 dilution but none completely inhibited lysis in the 1:16 dilution. The results were remarkably uniform in contrast with comparable inhibition tests using cattle serum as inhibitant. (Some J-positive cattle serums show only partial inhibition in the 1:1 and 1:2 dilutions in contrast with some which show complete inhibition through dilutions as high as 1:256.) Serums of 29 J-positive and 29 J-negative rabbits were examined for presence of natural agglutinins for human red cells. Fifteen of the J-positive rabbits possessed no agglutinins for human red cells whereas the remaining 14 possessed certain weakly expressed agglutinins which reacted approximately equally with all 4 groups. Only 3 of the J-negative serums failed to agglutinate human red cells. Seven had rather weakly expressed agglutinins reactive only with group A and AB red cells; 11 had weak agglutinins reactive with group A, AB and B red cells, whereas 8 had weak agglutinins which reacted with the red cells of all 4 groups. In the latter 2 classes, however, reactions with A and AB red cells were usually more intense than were those with cells of group B or group O.

In view of the indication of natural anti-human A agglutinins in the majority of the J-negative rabbits but in none of the J-positive rabbits, we are of the opinion the J classification described here parallels the classification(6,7) of "A-positive" and "A-negative" rabbits by more difficult technics involving both complement fixation and inhibition. We did not attempt to confirm this opinion by use of those technics(6,7). Nevertheless, we note at this point a further parallel, *i.e.*, that it was only the J-negative rabbits of the present study, like the A-negative rabbits of previous studies (see 7), which produced strongly reactive agglutinins specific for A of man on immunization with group A red cells.

In Table I are summarized the results of titration of 27 antisera, engendered by immunization with human group A red cells, in tests with J-positive and J-negative cattle bloods. Six of 12 antisera (including 2 obtained following booster injections) produced in J-positive rabbits contained no hemolysins for cattle red cells, whereas the remaining 6 contained some hemolysins (titers of 1:4 to 1:256) which failed to distinguish between J-positive and J-negative cattle as confirmed by absorptions. On the other hand, 14 of 15 antisera (including 4 obtained following booster injections) produced in J-negative rabbits contained hemolysins which cross reacted specifically with the red cells of J-positive cattle as indicated by the marked differences in titer shown in Table I. In fact, 6 of the antisera had titers ranging from 1:64 to 1:2048 with J-positive red cells but did not cross react at all with J-negative red cells. In any event, the lysins which did cross react with J-negative bloods (antiserums numbered RH28, 31, 33, 35 and 37) could be readily removed by absorption with J-negative bloods without appreciably affecting either degree of reactivity or titer of the antisera in tests with J-positive bloods. Conversely, however, J-positive bloods absorbed all the hemolysins

TABLE I. Cross Reactions of Rabbit Antisera against Human Red Cells of Group A in Lytic Tests with J-Positive and J-Negative Cattle Red Cells.

Antiserum No.*	"J" classification of rabbit	Lysins for cattle in normal serum and their titer	Antiserum titer in tests with cattle red cells: J-pos	J-neg
RH 9	J-neg	Yes,† 4	512	0
RH10	"	None	64	0
RH11	"	"	0	0
RH12	J-pos	"	32	32
RH13	"	"	0	0
RH14	"	"	0	0
RH15	"	"	0	0
RH16	"	"	0	0
RH17	J-neg	"	64	0
RH18	J-pos	"	4	4
RH19	"	"	0	0
RH28	J-neg	"	256	16
RH29	J-pos	"	0	0
RH30	"	"	32	32
RH30 #2			128	128
RH31	J-neg	Yes,† 16	256	16
RH32	J-pos	None	16	16
RH32 #2			256	256
RH33	J-neg	Yes,‡ 4	256	16
RH33 #2			2048	0
RH34	J-neg	Yes,† 16	256	32
RH35	"	" , 4	64	8
RH35 #2			256	16
RH36	J-neg	None	1024	0
RH36 #2			1024	0
RH37	J-neg	"	512	64
RH37 #2			2048	32

* #2 indicates antiserum of secondary series.
† Species heterolysins.
‡ Lysins specific for the A factor of cattle.

for J-negative bloods, thereby indicating that all cattle have at least one common blood factor which, like blood factor J (see 1), is serologically related to the A substance of man.

Tests with several of the antisera (RH9, RH36 and RH37 #2 of Table I) have now been performed in parallel with our best J reagents (C86 and C100) obtained from normal cattle serum and also our best J antiserum obtained by immunizing J-negative cattle with human red cells of group A. In general, RH9 and RH36 antisera proved to be equivalent or nearly so to the C100 J reagent and to the J antiserum produced by immunizing a J-negative heifer with group A red cells. They closely approached but did not quite measure up to the C86 reagent in ability to lyse weakly reactive J-positive bloods. On the other hand, the RH37 #2 antiserum has so far proved to be superior to all of our other J reagents in its ability to lyse the red cells of weakly reactive J-positive bloods. Whether this reagent will lyse the red cells of all truly J-positive bloods cannot, of course, be proved. Nevertheless, we have yet to find J-positive cattle blood, as measured by inhibition tests, in which the red cells cannot be lysed by the combined action of RH37 #2 reagent and guinea pig complement. This is the more remarkable considering that the RH37 #2 reagent was used in dilutions of 1:50 and 1:100.

J-positive and J-negative rabbits were also immunized with a variety of other bloods including human red cells of groups B and O, sheep red cells of groups R and O, and both J-positive and J-negative cattle and goat blood. With the exception of 2 out of 4 antisera produced in J-negative rabbits by immunizing with red cells of group R sheep, there was no indication of antibodies which would differentiate the red cells of J-positive from J-negative cattle. The J hemolysins in the 2 rabbit anti-sheep R serums were considerably weaker than those engendered by human red cells of group A, but like the latter were most effective with guinea pig complement.

Summary. It is shown that certain antibodies engendered in "J-negative" (or A-negative) rabbits by immunizing with human red cells of type A will selectively lyse red cells of J-positive cattle. Some of the "J" reagents so produced have proved to be either equal to or superior to the better J reagents obtained from J-negative cattle in their ability to promote lysis of the more weakly reactive intergrades of J-positive blood. The key to recognition of these specific cross reactions was substitution of guinea pig complement for rabbit complement. The method of classifying J-positive and J-negative rabbits is described and data are presented on distribution of the J types in 110 rabbits.

We are indebted to the Sacramento Medical Foundation Blood Bank for human blood used in this study.

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Effects of Antiserum in Bioassay of Thyrotropin and Thyroid Activator of Hyperthyroidism. (26032)

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Recently evidence has accrued that there is in the blood of patients with Graves' disease a substance which can stimulate the thyroid gland of guinea pig(1,2), mouse(3), and presumably the patient's own gland. It has been suggested that this substance may not be thyrotropin of pituitary origin(4); this was based on several qualitative differences shown to exist between the activity found in the blood in hyperthyroidism and that found either in pituitary extracts or in the blood of patients with myxedema. A further distinguishing feature is furnished in the present study, wherein antisera to bovine (pituitary) thyrotropin, produced in rabbits, were found to inhibit thyrotropic activity of bovine pituitary extracts and that in the blood of patients with myxedema, but not the thyroid activator in serum of patients with hyperthyroidism.

Methods. Antiserum (A) to commercial bovine thyrotropin (thyrotron, Nordic Biochemicals) was prepared by administering intramuscularly, 3 weeks apart, 2 injections of 2.5 mg of this preparation emulsified in Freund's adjuvant (complete), to totally thyroidectomized rabbits. Antiserum (B) to U.S.P. standard (bovine) thyrotropin was prepared by administering intradermally 5 weekly injections of 1 mg of this preparation emulsified in Freund's adjuvant (complete), in the interdigital webs of intact rabbits. These antisera were tested for titer and specificity using the bis-diazotized benzidine hemagglutination test, as previously described (5,6,7).

Thyrotropic effect was assayed by a method previously described(8); mice with thyroidal iodine labelled with I^{131} were injected intravenously with serum or other test solution and response measured as an increase in circulating radio-activity. This was expressed as a percentage of pre-injection concentration of I^{131} in the blood and a positive response was thus greater than 100%. Four to 6 mice were injected with each test solution. Thyrotropin of pituitary extracts or that found in the blood in myxedema produced maximal effect within 2 hours, but the thyroid activator in the blood in hyperthyroidism had maximal effect only after about 9 hours(3).

Human serum for assay was obtained from 3 patients with spontaneous myxedema and 3 with hyperthyroidism (Graves' disease). Diagnosis had been made previously by conventional criteria of physical examination and measurement of protein bound iodine in serum and thyroid uptake of I^{131}. Serum was separated from the blood within an hour of its being obtained, and stored at 4°C until used for assay, within one week in all instances.

Mixtures of serum or thyrotropin standard (U.S.P.) with antiserum or control serum were kept at room temperature for 30 minutes then centrifuged. Although no gross precipitate was seen the upper portion of fluid in the centrifuge tube was always used for assay.

Results. The hemagglutinating antibody titer of these thyrotropin antisera ranged

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TABLE I. Inhibition of Standard (Pituitary) Thyrotropin by Antiserum.

Exp.		Preparation assayed*	Ratio	Vol of inj. (ml)	Response§
I.	a.	Antiserum A + saline†	1:1	.5	213 ± 97
	b.	" " + .5 mu thyrotropin in saline	1:1	.5	376 ± 73
	c.	Human serum (normal) + .5 mu thyrotropin in saline	1:1	.5	333 ± 13
	d.	" " " + saline	1:1	.5	60 ± 6
Comment:		Antiserum A contained antithyrotropic activity (b less than a + c) simultaneously with thyrotropic activity.			
II.	a.	Antiserum A + .5 mu thyrotropin in saline	1:10	.5	107 ± 39
	b.	" " + .5 mu *idem*	1:100	.5	246 ± 84
	c.	" " + .5 mu "	1:1000	.5	293 ± 88
Comment:		Thyrotropic effect diluted out by 10 parts saline, but antithyrotropic activity retained; latter diluted out by 100 parts saline.			
III.	a.	.5 mu thyrotropin in saline	—	.25	452 ±182
	b.	Antiserum A + .5 mu thyrotropin in saline	1:10	.25	119 ± 39
	c.	N.R.S.‡ + .5 mu *idem*	1:10	.25	399 ± 92
	d.	Antiserum A + 2.0 mu "	1:10	.25	264 ± 75
Comment:		.5 mu thyrotropin inhibited by antiserum diluted 1:10; 2.0 mu thyrotropin partially inhibited by antiserum diluted 1:10 (assuming that 2.0 mu should have produced a greater response than .5 mu(8)).			
IV.	a.	.25 ml N.R.S. inj. i.v. before .5 mu thyrotropin in .2 ml saline	—	—	240 ± 27
	b.	.25 ml antiserum B inj. i.v. before .5 mu thyrotropin in .2 ml saline	—	—	80 ± 28
Comment:		Inj. of antiserum B inhibited effect of a subsequently inj. dose of thyrotropin.			

Thyrotropin was assayed as described under *Methods.* Control (*i.e.* inactive) injections gave a response of 97 ± 4 (stand. dev.)(8).

* See methods. † Saline = .9% (w/v) sodium chloride in water. ‡ N.R.S. = normal rabbit serum. § Mean ± stand. dev. of responses in 4 to 6 mice in each instance.

from 1/4000 to 1/12,000. Both antisera showed extensive cross reactions with other pituitary hormones, namely, corticotropin, growth hormone and gonadotropin. However, a hundred-fold increment in quantity of any of these hormones, as compared with either thyrotropin preparation, was required to produce a definite antigen-antibody reaction (hemagglutination-inhibition). Both thyrotropin preparations had similar quantitative and qualitative properties in this respect.

Antiserum A was found to contain both thyrotropic and antithyrotropic activity (Exp. I, Table I); only the latter remained in evidence when one volume of antiserum was diluted with 10 volumes of physiological saline. With further dilution (1:100) neither antithyrotropic nor thyrotropic effect was detected (Exp. II, Table I). No thyrotropic effect was shown with antiserum B. Although precise quantitation was not attempted it seems from Exp. III (Table I) that 0.023 ml of antiserum A could inactivate from 0.05 to 0.2 mu. of thyrotropin. In Exp. IV, (Table I) injection of the two substances separately still resulted in inhibition of thyrotropic effect.

With serum from patients with myxedema, endogenous thyrotropic activity was completely inhibited by prior incubation with antiserum added in a ratio of 1 to 10 volumes of human serum (Fig. 1, a). Similar assays carried out with serum from patients with hyperthyroidism (Fig. 1, B) failed to show significant inhibition of the thyroid activator contained therein. In the case of the first 2 sera from hyperthyroid patients 1 part of antiserum was added to 10 parts of human serum; in the third assay (responses 1300 and 1290) antiserum B and human serum were mixed in equal parts.

Discussion. Antisera to commercial and U.S.P. standard thyrotropin preparations had similar immunologic properties. While neither antiserum was specific for thyrotropin, the quantitative serological data suggest that the major antibody in both was probably to

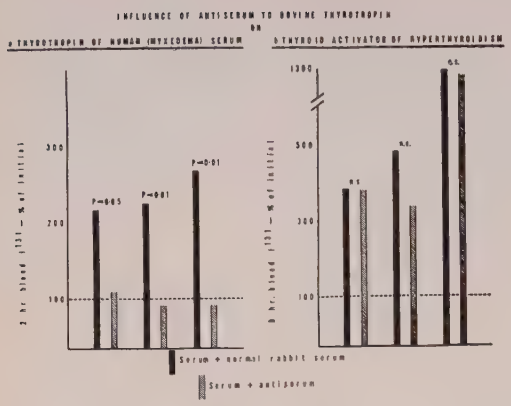

FIG. 1. Horizontal interrupted lines indicate mean values obtained with control studies(3,8). Quantities of normal rabbit serum used in the experiments, when assayed separately, gave negative responses both 2 hr and 9 hr after inj. Significance of the difference of mean responses was established by Student's "t" test.

thyrotropin itself. The cross reactions between thyrotropin antisera and the other pituitary hormones doubtless reflect the impurity of the thyrotropin preparations.

These antisera were shown to inhibit the biological effect of thyrotropin and this was brought about either by preincubation of antiserum with thyrotropin or by separate injection of the two substances. This is in agreement with work reported many years ago(9) and more recently(10,11). Inhibition of thyrotropin in the serum of patients with myxedema by the antisera infers a degree of immunochemical similarity between bovine thyrotropin and human thyrotropin; this was shown also in similar studies using human pituitary extract(11). That the thyroid activator in the blood of the 3 patients with hyperthyroidism was not inhibited infers, on the other hand, that this substance is qualitatively different from thyrotropin. This would be in agreement with other qualitative differences previously described(4).

The findings with serum from patients with Graves' disease are, however, at variance with those reported by Werner and his colleagues (11). They described that the globulin fraction from rabbit antiserum to thyrotropin inhibited thyrotropic activity of an extract of serum from a patient with Graves' disease. The assay method used was similar to our own but response in the mice was measured only 2 hours after injection of the extract. This is considered to be the time at which maximal effect from thyrotropin is found, distinct from the time (7 to 9 hours) after which maximal effect from the thyroid activator of hyperthyroidism is found(3).

It is of interest that thyrotropic and antithyrotropic activity occurred simultaneously in antiserum A. In view of the concentration of thyrotropin reported(12) in normal rabbits, detection of the hormone in blood of thyroidectomized rabbits would be expected with the assay method used here(8). Perhaps this endogenous hormone was combined with antibody, and the complex, retaining thyrotropic activity, remained dissolved in presence of excess antibody.

Summary. Rabbit antisera to commercial and U.S.P. standard thyrotropin preparations were shown to inhibit biological assay of bovine pituitary thyrotropin. While similar inhibition of endogenous thyrotropin in serum from patients with myxedema was shown, the antisera did not inhibit the thyroid activator in blood of patients with Graves' disease. This constitutes further evidence for the dissimilarity of the thyroid activator of hyperthyroidism and thyrotropin.

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Relation of Age and Race to Serum Cholesterol Ester Fatty Acid Composition.*† (26033)

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The authors have shown(1) that there is a correlation between level of arachidonic acid in the serum cholesterol ester fatty acid (CEFA) fraction and species susceptibility to atherosclerosis. Those species (rat and dog) with a high level of arachidonic acid in their serum cholesterol esters are known to be resistant to production of atherosclerosis, while the more susceptible species (man, chicken, goose, rabbit and guinea pig) have low levels of that acid. Atherosclerosis starts to develop in man at a very early age(2) and does not reach its maximum severity until the fifth decade and beyond. It is claimed that during this period there is an increase in blood cholesterol level which may be related to development of the disease(3). Another factor may be the diet, which appears to bear a closer relationship to development of atherosclerosis than race(4). To provide more specific information regarding the suggested correlation of CEFA spectrum and susceptibility to atherosclerosis a study was carried out regarding relation of age and race to serum CEFA composition.

Methods and materials. All subjects reported in this study were males. The older subjects (negro and white), aged 60-87, were from the hospital medical wards. They were all in good nutritional status. The younger subjects (negro and white), aged 6-10 years old, were from an institution (D.C. Junior Village). Both age groups consumed the usual institutional diet containing approximately 30-35% fat. Fasting blood samples were obtained from the different subjects. Lipid extracts of the serum were prepared as previously described(5). Serum cholesterol levels were determined by the method of Sperry and Webb(6). Cholesterol esters were separated from the other lipid components by silicic acid chromatography(7). The isolated cholesterol esters were interesterified in HCl-methanol and methyl esters were sublimed according to the procedure of Stoffel *et al.*(8). Gas-liquid chromatography was carried out as previously described(9).

Results. The CEFA spectrum of the different groups is shown in Table I. In all subjects, regardless of age or race, the major fatty acid in the CEFA fraction of serum was linoleic acid (43.0-52.8%). Other fatty acids also occurred in substantial amounts: oleic (20.8-25.1%), palmitic (13.3-15.0%) and arachidonic acid (5.3-7.1%). Comparison of the different age groups, both negro and white, indicates that there were highly significant differences between the proportions of 2 of the fatty acids (oleic and linoleic) in serum cholesterol esters. Both negro and white children had significantly less oleic acid ($P<.01$) and significantly more linoleic acid ($P<.01$) than the older subjects. While older subjects had apparently higher percentages of arachidonic acid in their serum cholesterol esters, these differences were not considered significant since only values of $P<.01$ were considered significant in the present study. There were no significant differences between the groups in any of the other CEFA. Also, there were no significant differences in % oleic and linoleic acids of the CEFA between negro and white subjects of the same age group.

Table II shows blood cholesterol levels of the different subjects. Both negro and white children had significantly lower total blood cholesterols ($P<.01$) than older subjects. There were no significant differences in blood cholesterol levels of the 2 races.

Discussion. Our results clearly show that serum cholesterol esters of children have a significantly larger proportion of linoleic acid and less oleic acid than older individuals. At

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TABLE I. Relation of Age and Race to Serum Cholesterol Ester Fatty Acid Composition.

Fatty acid*		Negro†		White†	
Chain length carbons	No. double bonds	Avg age 7.7 yr (9)‡	Avg age 67 yr (13)	Avg age 8.5 yr (10)	Avg age 72 yr (13)
		% total fatty acids			
6 to 12		.5 ± .1§	1.2 ± .3	.5 ± .1	1.0 ± .2
14	0	.9 ± .2	1.0 ± .3	.9 ± .2	.9 ± .1
14	1	.2 ± .1	.4 ± .2	.2 ± .0	.4 ± .1
16	0	13.7 ± .9	15.0 ± 1.6	13.3 ± 1.1	13.8 ± 1.5
16	1	3.0 ± 1.0	4.2 ± 1.1	3.2 ± .4	4.4 ± 1.5
16	2	.3 ± .1	.3 ± .1	.4 ± .0	.4 ± .1
18	0	1.5 ± .5	1.3 ± .5	1.2 ± .4	1.5 ± .5
18	1	20.9 ± 1.2	25.1 ± 2.7	20.8 ± 1.4	24.9 ± 2.7
18	2	51.8 ± 2.5	43.0 ± 3.5	52.8 ± 1.8	44.7 ± 4.0
18	3	.5 ± .1	.5 ± .4	.5 ± .1	.6 ± .3
20	0	.4 ± .1	.5 ± .3	.6 ± .2	.5 ± .2
20	3	.3 ± .1	.4 ± .1	.3 ± .0	.4 ± .3
20	4	6.0 ± .5	7.1 ± 1.7	5.3 ± .9	6.5 ± 1.7

* Represents major fatty acids determined. Small amounts of others were also detected.
† All subjects were males.
‡ Figures in parentheses indicate No. of subjects.
§ Stand. dev.

TABLE II. Influence of Age and Race on Serum Cholesterol Level.

Avg age, yr*	Race	Cholesterol, mg % Free	Ester	Total	Ester/Total, %
7.7	Negro	40 ± 4	138 ± 12	178 ± 16	77.5 ± 1.3
8.5	White	40 ± 9	144 ± 28	184 ± 35	78.3 ± 3.3
67	Negro	63 ± 8	166 ± 26	229 ± 32	72.5 ± 2.3
72	White	61 ± 15	162 ± 35	223 ± 50	72.7 ± 1.4

* No. of subjects indicated in Table I.

the same time, younger individuals also have a lower blood cholesterol level. It might be suggested that level of linoleic acid in the serum CEFA fraction bears an inverse relationship to serum cholesterol. Of particular interest is the finding that there is very little change in % arachidonic acid in the serum CEFA with advancing age. The level of that acid in the serum CEFA fraction appears to be a characteristic of the species(1) and does not appear to be affected by age. This may also be related to the observation that man is susceptible to atherosclerosis and that the disease starts to develop at an early age. The question still to be answered is whether the arachidonic acid level of serum CEFA in man can be altered by experimental means.

Summary. The serum CEFA and cholesterol level of children (6-10 years old) and older subjects (60-87 years old) of both negro and white races have been compared. Children of both races had significantly less oleic acid and significantly more linoleic acid in their serum CEFA fraction than older subjects. Arachidonic acid did not show significant changes with increasing age. Negro and white subjects of the same age group did not show significant differences in CEFA spectrum. Also, children had a significantly lower total blood cholesterol level than older individuals. The significance of these findings as related to atherosclerosis is discussed.

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Identification of An Antigen Common to *Listeria monocytogenes* and Other Bacteria.* (26034)

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An antigen common to Gram-positive bacteria was described by Rantz and associates (1,2) and referred to as non-species specific antigen. Subsequent studies revealed that this heterogenetic antigen is present in many but not all species of these bacteria. For example, it was not detected in *Staphylococcus citreus* and *Micrococcus lysodeikticus*, even after lysis of the latter microorganisms by lysozyme(3,4). The antigen is produced by certain but not all species of the genus *Bacillus* (5). The present study was undertaken to determine whether *Listeria monocytogenes* produces this antigen and, also, whether it is this antigen that is the basis for the serologic crossreactions between this microorganism and *Staphylococcus* described by Seeliger(6) and Welshimer(7).

Materials and methods. Strains of *L. monocytogenes* (types 1, 2, 3, 4a, 4b) were received through the kindness of Dr. J. T. Barrett, Univ. of Missouri, and Dr. M. L. Gray, Montana State College. The former strains were obtained from CDC (KC 222 to 226). Dr. Gray's strains types 1 and 4a originated from the National Type Culture Collection (#5349 and #5214); types 2 and 4b from Dr. Donker-Voet, Utrecht, Netherlands; type 3 was isolated from a fatal case of meningitis in Muskegon, Mich. Dr. H. J. Welshimer made available one strain each of *L. monocytogenes* type 1 and *Staphylococcus epidermidis*. Strains of *S. aureus* and *Bacillus subtilis* were isolated in this laboratory, *S. aureus* strain D was obtained from Difco Laboratories, Detroit. The strains were maintained on blood agar.

For preparation of antigens, the microorganisms were grown on brain veal agar in Kolle flasks at 37°C for 18 hours. To each flask was added 20 ml of phosphate buffer of pH 7.3. The suspensions were centrifuged immediately at 23,500 G for 20 min in a refrigerated centrifuge. Supernates of infusion broth cultures were also used. Supernates, notably of type 4a, proved to be hemolytic; it was observed that heating at 56°C for 15 min abolished this effect. Therefore, the antigens were routinely heated and then kept at 4°C until used.

The hemagglutination test was carried out as previously described(3,8). Briefly, human red blood cells (blood group O) were washed 3 times, mixed with antigen in suitable dilutions, and incubated for 30 min at 37°C. The modified erythrocytes were washed 3 times and then used in the hemagglutination test. Antiserum in serial 2-fold dilutions (vol. 0.2 ml) was mixed with 0.2 ml of the modified red blood cell suspension. The mixtures were incubated in a waterbath at 37°C for 30 min, and the resulting hemagglutination was read grossly after centrifugation at 1300 G for 3 min. *L. monocytogenes* antisera were obtained from Dr. Gray. Others were prepared in this laboratory by injection, 2 days apart, of suspensions of agar grown bacteria (2 ml) that had been heated at 56°C for 15 min, followed one week later by injection of unheated suspensions. The animals were bled one week after immunization.

* Study aided by research grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

TABLE I. Heterogenetic Antigen in *Listeria monocytogenes*.

	Antigens						
	Listeria monocytogenes Types					*Staphylococcus*	*B. subtilis*
Staphylococcal antiserum	1	2	3	4a	4b		
	Hemagglutination titers (reciprocal)						
Pre-immunization	<100	<100	<100	<100	<100	100	<100
Post-immunization	25600	12800	12800	12800	12800	25600	25600
Absorbed with							
L. monocytogenes (all types)	<400	<400	<400	<400	<400	<400	<400
B. subtilis	<400	<400	<400	<400	<400	<400	<400
Staphylococcus (D)	<400	<400	<400	<400	<400	<400	<400

Staphylococcal rabbit antiserum, prepared by injection of viable bacteria (strain D), had a hemagglutinin titer of 1:12,800 and failed to agglutinate (in titer of 1:200) erythrocytes modified by *Salmonella, Shigella,* and *Escherichia coli* antigens. Another staphylococcal antiserum was obtained from rabbits after injection of antigen A, kindly supplied by Dr. K. Jensen(9). Normal rabbit sera were employed for control purposes.

Absorption tests were carried out by mixing the sediment of a washed bacterial suspension (5 ml) with antiserum (2.5 ml) in the appropriate dilution. The mixtures were incubated in a waterbath at 37°C for 30 min. and the supernates, together with appropriate controls, were titrated in the hemagglutination test. Hemagglutination inhibition tests were performed by mixing antiserum (0.2 ml) in serial 2-fold dilutions and supernates of bacterial suspensions (0.1 ml), incubating the mixtures at 37°C for 30 min and adding antigen-modified erythrocytes for demonstration of remaining antibodies.

Results. Hemagglutination tests for detection of heterogenetic antigen in *L. monocytogenes* were carried out with staphylococcal antiserum. For control purposes the preimmunization serum was used. The results of a typical experiment recorded in Table I show that the antiserum in high dilution caused agglutination of erythrocytes modified by the antigens of all types, in contrast to the preimmunization serum. It can be seen also that the antibody titer of the serum against *L. monocytogenes* is of the same order of magnitude as that against the unrelated bacterial species. Additional experiments with 6 different strains of the identical types yielded the same results.

Maximal hemagglutination titers were obtained with dilutions of supernates of 1:2 to 1:5, and minimal hemagglutination occurred with antigen dilutions up to 1:160. Heterogenetic antigen reactive with staphylococcal antiserum was detected also in broth cultures of all 11 strains. It is noteworthy that types 4a and 4b produced more of this antigen in broth than on agar, as revealed by quantitative titrations of the supernates.

Additional experiments showed that hemolysis results when sheep red blood cells are modified by heated *L. monocytogenes* antigen and mixed with either *Staphylococcus* or *L. monocytogenes* (rabbit) antiserum and guinea pig complement. No striking difference in sensitivity was found between hemagglutination and hemolysis tests.

Further evidence of the identification of the antigen was obtained by means of absorption tests. As shown in Table I, *S. aureus, B. subtilis,* and *L. monocytogenes,* all remove at least 96% of the antibodies from the staphylococcal antiserum. Similar results were obtained in hemagglutination-inhibition tests.

The specificity of the hemagglutination test is shown also by the results of experiments with a strain of *S. aureus* and 2 mutants derived therefrom (ATCC #13679 to 13681), kindly made available by Dr. J. A. Di Paolo of the Roswell Park Memorial Institute, Buffalo. Supernates of the parent strain contained large amounts of antigen, demonstrable by hemagglutination with staphylococcal antiserum, in contrast to those of the mutants.

Further, only the supernate of the parent strain inhibited agglutination by *S. aureus* and *L. monocytogenes* antisera of erythrocytes modified by either *B. subtilis* or *L. monocytogenes* antigens. These observations may conceivably aid investigators in studies of the enzyme systems responsible for the synthesis of this heterogenetic antigen.

If, as indicated by these experiments, *L. monocytogenes* contains heterogenetic antigen, then, antibodies against this antigen should be present in *L. monocytogenes* antisera. Titration of these antisera revealed that all agglutinated erythrocytes modified by either *L. monocytogenes, S. aureus,* or *B. subtilis* antigens. Of Dr. Gray's antisera those against types 1 and 2 had a higher titer (1:1600 to 1:3200) than those against types 3, 4a, and 4b (1:400 to 1:800), when titrated in *Staphylococcus* or *B. subtilis* hemagglutination tests. Dr. Welshimer's type 1 antiserum had a titer of 1:200. Sera from 7 normal rabbits did not contain these antibodies in titers of 1:100 or higher. The antisera against *L. monocytogenes* types 1, 2, and 3 prepared in this laboratory by immunization with viable bacterial cells contained antibodies in titers of 1:12,800 to 1:25,600. Antisera prepared by a single injection of viable bacteria of types 4a and 4b contained antibodies in titers of 1:400 to 1:800. In contrast, the preimmunization sera had titers of less than 1:50. Additional studies revealed that the antibodies of all these antisera were removed by absorption with *B. subtilis*. Differences in antigen preparations used for immunization (viable, formalinized, autoclaved, etc.) and/or immunization schedule may account for differences in titers of antibodies against this heterogenetic antigen. From the data presented here it is concluded that *L. monocytogenes,* types 1, 2, 3, 4a, and 4b, contain heterogenetic antigen in common with *S. aureus* and *B. subtilis*.

Discussion. Recent investigations indicate that listeriosis of man is more common than was hitherto recognized. For serologic identification of the pathogen and for studies of antibody response of patients as a tool for immunologic diagnosis of this infection knowledge of the antigenic composition of the microorganism is indispensable. In the present studies it has been shown that *L. monocytogenes* contains a heterogenetic antigen in common with *S. aureus* and *B. subtilis*. The antigen is present in supernates of agar and broth grown strains of all types and can be detected by means of a hemagglutination test. The antigen readily becomes attached to human erythrocytes and is heat stable (15 min at 100°C). It appears to be related to, or identical with, the antigen described by Rantz and associates(1,2) in many other species of Gram-positive bacteria.

Serologic crossreactions between *L. monocytogenes* types 1, 2, and 3 and *S. aureus* were previously recognized by Seeliger and Sulzbacher(6). Welshimer(7) observed that rabbits which had been immunized with *S. epidermidis* and, after a rest period of some 100 days, challenged with *L. monocytogenes* responded with production of staphylococcal antibodies in high titer. Conversely, *S. epidermidis* elicited a booster effect in animals previously injected with *L. monocytogenes*. It may be added that the strain of *S. epidermidis* used by Dr. Welshimer was shown in this laboratory to produce substantial amounts of this antigen. The findings described here indicate that the *L. monocytogenes* antigen is not species specific but of the Rantz type. It can be anticipated that a similar or identical antigenic relationship can be demonstrated between *L. monocytogenes* and many other bacterial species, for this heterogenetic antigen is produced by hemolytic streptococci of groups A, B, and C; enterococci; viridans streptococci; pneumococci; *B. megaterium, B. cereus,* and others. Seeliger(10) previously found a common antigen in enterococci and *L. monocytogenes* As yet the chemical composition of the antigen of the Rantz type is unknown.

Cognizance of the existence of heterogenetic antigens in various bacterial species is indispensable for critical utilization of all serologic methods, including the fluorescent antibody technic. In this connection it should be emphasized that human sera frequently contain antibodies against the heterogenetic antigen of the Rantz type(11). This antibody readily passes the placental

barrier and thus is present in newborn infants. The titer of the antibodies decreases during the first 3 months of life and later increases, so that older children almost invariably have this antibody. The results shown here suggest that for demonstration of species specific *Listeria* antibodies in either animal or human serum absorption with *S. aureus* or *B. subtilis* be considered to eliminate heterogenetic antibodies of the Rantz type. The absorption experiments recorded here failed to make possible the detection of species specific antibodies in *L. monocytogenes* immune sera by means of the hemagglutination test used in this investigation.

Summary. *L. monocytogenes* (types 1, 2, 3, 4a, and 4b) produces a heterogenetic antigen demonstrable by hemagglutination test with suitable antisera. Sheep erythrocytes modified by this antigen are lysed in presence of (rabbit) antiserum and guinea pig complement. Antibodies against this antigen are present in certain *L. monocytogenes* and *S. aureus* (rabbit) antisera and can be removed by absorption with either *L. monocytogenes* (of all types), *S. aureus,* or *B. subtilis.* The findings may help to avoid a possible pitfall in interpretation of serologic tests for species specific *L. monocytogenes* antibodies.

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Vibrio-like Bacteria Recovered from Cultures of "*Spirochaeta myelophthora*."* (26035)

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In studies in this laboratory during the early months of 1958 which were unavoidably interrupted, 4 of 5 cultures obtained from Miss Rose Ichelson of St. Luke's and Children's Medical Center, Philadelphia, termed by her, following Steiner, "*Spirochaeta myelophthora,*" yielded vibrio-like bacteria. Ichelson(1,2) had implicated these microorganisms in multiple sclerosis. After Martin, *et al.*(3), published observations similar to those obtained in this laboratory, it seemed desirable to put these fragmentary findings on record. Included are cultures of 6 spinal fluid samples.

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Cultures. Two cultures originally from Ichelson were obtained from Dr. Albert Cohn of Montefiore Hospital, New York, labelled, and recoded as in brackets []: "0702 Subc 12-10-57 (1-2-58)"—[NYA]; "0793 Subc 11-21-57 from 11-9-57 Subc 1-2-58"—[NYB]. Three other cultures obtained directly from Ichelson's laboratory were "0778 Zeissman—11-7-57—14th subculture from 472"—[PC]; "0781 CH. Sol—11-7-57—6th Subc. 465"—[PD]; and "0791 A. M. 11-19-57—11th Subc. from 491"—[PE]. Ichelson's photomicrographs(1) labelled "case A. M." may correspond with PE; and the strain listed by Martin, *et al.*(3) as I472 may be the same as PC.

FIG. 1-5. Four-day cultures on sheep blood-HI streaked plates washed off with distilled water. Chromium shadowed. Electron micrographs courtesy of Dr. Bryce L. Munger and Dept. of Anatomy, School of Med., Washington Univ. Fig. 1 and 4, strain NYB; Fig. 2, strain PE; Fig. 3, strain PC; Fig. 5, strain PD.

Methods. The principal media used were Bacto heart infusion (HI) agar or broth or BBL trypticase-soy (TS) agar or broth, usually enriched with 5% of defibrinated sheep or human blood. Fermentation tests were performed with "Redi-discs" (Pennsylvania Biological Labs.) in TS broth containing 1% human serum and phenol red indicator, or with "Redi-discs" on streaked TS agar plates. TS broth or agar was used for other biochemical tests. Antibiotic sensitivity tests utilized discs on TS-sheep blood agar. Anaerobic conditions were obtained in Brewer jars using H_2 with Pt catalysis.

Results. Morphological study revealed forms similar to those described by Ichelson in all 5 cultures. Strain NYA was non-motile and failed to grow in all trials. Strain PE was found motile twice but grew irregularly and was not included in biochemical or antibiotic sensitivity tests. Electron micrographs were, however, obtained of this and of the other 3 strains (Fig. 1-5).

Poor growth or none appeared in cultures in media of the Ichelson type(1,2) including tubes supplied by Miss Ichelson and several variants prepared in this laboratory. In a routine check for purity, aerobic growth was obtained on HI-sheep blood streaked plates. The growth was compatible with the original descriptions, although the bacteria were clearly not spirochetes. These observations were repeated in several subsequent transfers from the original cultures. Strains NYB, PC and PD grew abundantly, usually on HI or TS agar plates with sheep blood. Human blood could be substituted, and satisfactory growth was also obtained with 1% human serum in TS broth, and in TS broth or on TS agar streaks without enrichment. Growth was more abundant or more consistent (a) at 30°C than at 37°; (b) aerobically than anaerobically (with 10% added CO_2); and

(c) aerobically without CO_2 than with 10% CO_2. Strain PE grew only on streaked plates of HI with sheep blood incubated at 30° aerobically with or without CO_2.

Colonies after 72 or 96 hours at 30° were less than 1 mm in diameter, grayish, translucent or transparent droplet-like, viscid. The organisms were Gram-negative; carbol fuchsin was more satisfactory as counterstain than safranin. Under phase-contrast or darkfield illumination, 4-day cultures showed thin, straight, crescentic or S-shaped forms with scattered attached or unattached protoplast-like bodies. Spirillar forms with 2 to 5 turns were present but not numerous; they appeared more frequently in strain PD than in the others. Electron micrographs of all 4 strains disclosed single polar flagella (Fig. 1-5).

Fermentation tests in broth with strains NYB, PC and PD showed neither acid nor gas production, in the presence of abundant growth, in glucose, sucrose, lactose, mannitol, maltose, galactose, inulin, xylose, trehalose, rhamnose, dulcitol, arabinose, fructose, salicin, inositol, melibiose, raffinose or starch. Similar results were obtained on streaked plates except that glucose was acidified by strains PC and PD at 48 hours (30°) but realkalinized at 96 hours. Strain NYB remained negative with glucose and all 3 strains showed no activity with the other substrates. The 3 strains failed to produce indol or H_2S or to liquefy gelatin; all 3 reduced NO_3 and gave weakly positive catalase and strongly positive oxidase reactions.

Antibiotic sensitivity tests (with Bacto discs except as noted) showed well marked inhibition of growth of all 3 strains by tetracycline, chlortetracycline, oxytetracycline and chloramphenicol (all 5 μg), by erythromycin (2 μg) and by matromycin (BBL, 10 μg). Penicillin (2 units) and dihydrostreptomycin (2 μg) inhibited strains NYB and PC but not PD; polymyxin B (5 μg) was only slightly inhibitory to strains NYB and PC and not at all to strain PD.

Six samples of spinal fluid provided by Dr. Irwin Levy and Dr. Leonard Berg, Dept. of Neurology, Barnes Hospital, were centrifuged at 1500 rpm for 2 hours and the sediment in approximately 1.5 ml of fluid inoculated, in 0.3 ml amounts, into Seitz-filtered Ichelson medium with and without petrolatum seals and into 5% sheep blood-HI and –TS broth; 0.1 ml amounts were streaked on 5% sheep blood-HI and –TS agar plates. All cultures, together with the supernatant spinal fluids, were incubated at 30° in air. Gram stains and fresh preparations examined by darkfield of the sediment at time of inoculation and after incubation for 5, 7, 14 and 46 days, were all consistently sterile. According to Dr. Levy, 5 of these samples were from patients with multiple sclerosis; one was from a normal control.

Discussion. Information available when these studies were made suggested that the organism described is a vibrio, more closely related to *Vibrio fetus* than to other species listed in the 7th edition of Bergey's Manual (4). *V. fetus* is said to differ in having an optimum growth temperature of 37°C, a preference for 10% CO_2 in the growth atmosphere, and in producing "no change or slightly acid" from lactose and sucrose as well as from glucose. Reich, Morse and Wilson (5) have reported that added CO_2 is not essential for the growth of *V. fetus*. King(6), who identified *V. fetus* recovered from infections in man, noted that it is oxidase-positive. The 3 actively growing strains of the present study were sent to Miss King, who found them different from *V. fetus* in their growth characteristics and stated that they were non-reactive in *V. fetus* antisera (personal communication, 1958). Martin, *et al.*(3) have since identified a group of cultures they obtained from Ichelson, one given them by Myerson, *et al.*(7), and 2 isolated from distilled water by Martin and his colleagues, with *Vibrio tyrogenus*, a type culture of which (A. T.C.C. No. 7085) was found to have essentially the same characteristics. *V. tyrogenus* is listed in the 6th(8) but not in the 7th edition of Bergey's Manual. The test data given in common by Martin, *et al.* and in the present report are in agreement.

Summary. Of 5 cultures named *"Spirochaeta myelophthora,"* received directly or indirectly from the laboratory of Miss Rose Ichelson in Philadelphia, 4 could be grown

on standard aerobic media and appeared as monotrichate polar-flagellated vibrio-like bacteria. Three that grew abundantly manifested characteristics that suggest relationship to but not identity with *Vibrio fetus*. Cultures of 6 spinal fluid samples, 5 from multiple sclerosis, remained sterile during 46 days.

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Response of Cartilage Sodium to Changes in Extracellular Fluid.* (26036)

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In recent years a number of investigators have explored the possibility that bone could serve as a reservoir for sodium. (For references see(1).) Infant skeleton differs from adult in that a sizeable portion (up to 30%) of the skeletal mass is composed of cartilage, and it is estimated that as much as one-third of total skeletal sodium in infants is present in the cartilaginous portion(2). It was of interest, therefore, to determine whether or not sodium content of cartilage would change with changes in serum sodium concentration.

Methods. Rabbits between 6 and 10 weeks of age were used. Anesthesia consisted of Dial with Urethane (Ciba) in dose of 0.7 cc/kg body weight. In the *control* series, opposite pieces of costal cartilage were removed following a 2-hour period of anesthesia. In the *hyponatremic* series, one cartilage sample was removed after 2 hours of anesthesia, and at the same time a sample of venous blood was obtained. Immediately following this procedure, a sodium-free isosmolar solution (Table I) equal in volume to 20% of body weight of the animal was injected intraperitoneally. Four hours later, a second blood sample was obtained, and the opposite piece of costal cartilage removed. In the *hypernatremic* series, initial blood and cartilage samples were obtained as above, followed by intravenous infusion of fluid of high sodium content (Table I) in an amount equal to 5% of the body weight. Infusion time varied from 45 to 180 minutes. Thereupon a second sample of cartilage and of blood was obtained.

Upon removal from the animal, the cartilage samples were immediately stripped of perichondrium and placed in tared stoppered weighing bottles. Cartilage samples averaged 20 mg in weight.

Cartilage water was determined by drying overnight in a vacuum oven at 70°C. The samples were then digested with concentrated nitric acid (0.1-0.3 cc) on a steam bath, and resulting solutions made up to volume for sodium analysis by flame photometry and chloride analysis by Volhard titration. Serum analyses were made by standard methods, whole blood pH by glass electrode.

Results. Control series. In 39 experiments, sodium content of opposite pieces of costal cartilage was determined. The mean difference in sodium content was 0.0082 meq/g wet weight, and standard error 0.0016 meq/

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TABLE I. Composition of Infused Solutions.

	mM/liter						g/liter	
	NaCl	$NaHCO_3$	KCl	$CaCl_2$	KH_2PO_4	$MgSO_4$	Sucrose	Glucose
Solution I*	—	—	3.5	5.1	1.2	2.5	96.16	1.00
" II†	600	300	3	5	1	3	—	—

* Equiosmolar with serum.
† Designed to give sodium load of 45 meq/kg body wt.

TABLE II. Results of Hyponatremia Experiments.

	Pre-infusion			Post-infusion		
Cartilage Na, meq/g	46*	.292†	(.245- .349)‡	46*	.253†	(.214- .305)‡
" H_2O, %	37	69	(63 - 75)	37	69	(60 - 73)
Serum Na, meq/l	46	150	(139 -168)	45	106	(73 -131)
" K, "	13	3.6	(2.0 - 4.4)	5	5.5	(4.4 - 6.2)
" Cl, "	13	96	(83 -103)	5	71	(60 - 78)
" CO_2 content, meq/l	13	31	(25 - 40)	5	22	(20 - 25)
Whole blood pH	20	7.38	(7.26 - 7.64)	6	7.23	(7.01 - 7.32)
Hematocrit	21	42	(33 - 48)	11	43	(39 - 49)

* No. of samples. † S.E. difference = .0037. ‡ Range.

g. It was thus apparent that opposite pieces of costal cartilage could be used with one serving as control for the other.

Hyponatremic series. The results of these experiments are summarized in Table II. Intraperitoneal infusion of the sodium-free solution produced a profound fall in serum sodium and chloride concentrations. Carbon dioxide content and pH also fell, and there was a slight rise in serum potassium concentration. Cartilage water did not change. Average cartilage sodium content fell from a pre-treatment value of 0.292 to a post-treatment value of 0.253 meq/g wet weight, a decrease of 0.039 meq, or 13.3%. This decrease is approximately 10 times the standard error of the difference, and can thus be considered statistically significant. The 13% decrease in cartilage sodium content can be contrasted to the fall in serum sodium of 29%.

Hypernatremic series. The results are summarized in Table III. Serum sodium rose as did serum chloride and carbon dioxide content. There was a slight fall in blood pH. The magnitude of the induced hypervolemia is evident from the marked fall in hematocrit. Average cartilage sodium content increased from a pre-treatment value of 0.291 to a post-treatment value of 0.323 meq/g, an absolute increase of 0.032 or 11%. This increase can be considered statistically significant inasmuch as it is approximately 7 times the standard error of the difference. The 11% increase in cartilage sodium can be contrasted to the 39% increase in serum sodium concentration.

Discussion. It is evident that changes in serum sodium concentration were associated with a change in cartilage sodium content under the conditions of these experiments. However, the magnitude of these changes could not be correlated with the magnitude of the change in serum sodium, nor degree of acidosis produced. The observed changes in whole blood pH may reflect inadequate ventilation

TABLE III. Results of Hypernatremia Experiments.

	Pre-infusion			Post-infusion		
Cartilage Na, meq/g	28*	.291†	(.241- .330)‡	28	.323†	(.297- .360)‡
" H_2O, %	28	69	(63 - 73)	28	69	(64 - 73)
Serum Na, meq/l	28	145	(132 -157)	28	202	(179 -273)
" K, "	28	3.8	(2.5 - 5.7)	28	3.4	(1.8 - 5.6)
" Cl, "	28	96	(84 -103)	28	142	(125 -183)
" CO_2 content, meq/l	28	32	(24 - 40)	28	38	(30 - 62)
Whole blood pH	27	7.38	(7.31 - 7.52)	24	7.29	(7.08 - 7.45)
Hematocrit	27	42	(36 - 49)	27	20	(16 - 32)

* No. of samples. † S.E. difference = .0046. ‡ Range.

or circulatory changes due to the large volumes of fluid infused.

It should be noted that the reduction in cartilage sodium (13%) was larger than we have ever been able to produce in bone(1), although others have found greater changes in response to acidosis and/or salt depletion. Furthermore, we have not been able to produce an increase in bone sodium by infusing sodium-containing solutions, though an increase amounting to about one-half that which we found to occur in cartilage (11%) has been observed by others following chronic sodium loading(3). In this respect the infant skeleton by virtue of its cartilage component would be expected to provide a larger reservoir of sodium relatively speaking, than that of the adult.

An attempt was made to find out whether the observed changes in cartilage sodium could be accounted for in the chloride space of this tissue. Eight samples of rabbit costal cartilage were analyzed for chloride; average value was 0.039 meq/g (range 0.033 to 0.044). The calculated chloride space was therefore 35% of the wet weight, or about half of the total water.† In the hyponatremic series, the change which could be ascribed to that occurring in the chloride space is (.150-.106) x 0.35 = 0.015 meq Na/g, whereas the total observed change was 0.039 meq/g. For the hypernatremic series the change in chloride space sodium was (.202-.145) x 0.35 = 0.020 meq/g, whereas the total change was 0.032 meq/g. In the first instance chloride space sodium accounted for only 39% of the change, in the latter 62% of the total change. Thus the observed changes in cartilage sodium cannot be accounted for solely by "extracellular" fluid, but must have involved some alteration in the component of sodium outside the chloride space.

Recently Farber(4) reported studies similar to ours. Using the ear cartilage of rabbits he was able to show a decrease in cartilage sodium of 16% following induction of hyponatremia. Only 40% of observed change could be ascribed to the chloride space. He further demonstrated that cartilage which has been partly depleted of sodium took up radiosulfur (S^{35}) somewhat less readily, suggesting an alteration in chondroitin sulfate metabolism.

Summary. Induction of hyponatremia by intraperitoneal injection of a sodium-free solution resulted in an appreciable fall (13%) in sodium content of rabbit costal cartilage. Induction of hypernatremia by intravenous injection of a sodium-rich solution caused an 11% increase in cartilage sodium content. These changes cannot be accounted for solely in the chloride space of cartilage. About one-half of the change must be ascribed to alterations in that portion of the cartilage sodium which is outside of the chloride space. The observed changes are larger than we have been able to produce in bone.

† Cl space $= \frac{\text{Cartilage Cl}}{\text{Serum Cl}} \times 0.92 \times 0.95.$

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Separation of Specific Antigens of *Histoplasma capsulatum* by Ion-Exchange Chromatography.* (26037)

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Histoplasmin, the filtrate derived from prolonged growth of the mycelial phase of *Histoplasma capsulatum* in liquid synthetic medium, has been employed for many years as the antigen in complement fixation, precipitin, and collodion agglutination tests for histoplasmosis(1,2). Recently Heiner reported its use in the Ouchterlony technic(3), which appeared to be a more sensitive and specific test for the disease. He detected six different antigens in crude mycelial filtrates, but only one, isolated by continuous-flow electrophoresis and designated as "h" antigen, was specific for active histoplasmosis(4).

Since use of purified "h" antigen would appear to provide more significant information to the clinician than does crude histoplasmin in the gel-precipitin test now in routine use in this laboratory, methods were explored for simple, reliable separation of this fraction from the crude filtrate. The purpose of this paper is to describe the initial separation of "h" antigen from other antigens by means of diethylaminoethyl-cellulose (DEAE) chromatography of partially purified histoplasmin.

Materials and methods. Preparation of crude antigen. The histoplasmin used in this study was prepared from cultures of *H. capsulatum* No. 4894, obtained from the late Dr. Rhoda Benham of Columbia University. Liquid asparagine medium, 1500 ml, was dispensed in 3000-ml Erlenmeyer flasks and inoculated with several small pieces of mycelium from Sabouraud agar cultures in Petroff flasks. After incubation of the cultures at room temperature (25°C) in the dark for approximately 6 months, the mycelial mats were broken up and sunk by violent agitation of the flasks. The medium was separated from the mycelial mats by filtration through paper, and sterilized by filtration through a Hormann filter. 'Merthiolate' in a final concentration of 1:10,000 was added to the crude filtrate as preservative. In the agar gel-precipitin technic, the crude preparation was shown to contain, in terms of antiserum from a patient with culturally proven histoplasmosis, at least 2 antigens. These were precipitated from solution by half saturation with ammonium sulfate or by addition of 2 volumes of acetone.

Chromatography. DEAE-cellulose was prepared by the procedure of Peterson and Sober(5). A 1.2 x 45 cm column containing 3 g of adsorbent was equilibrated with 0.01 M pH 8.0 phosphate buffer as suggested by Fahey *et al.*(6). The sample of partially purified histoplasmin was dialyzed for 24 hours against the same buffer in a revolving dialyzer. Three ml of the clear, but highly colored, solution containing approximately 150 mg of protein were applied to the column and washed down with several 5 ml portions of buffer. Gradient elution with increasing molarity from 0.01 M to 0.30 M phosphate and decreasing pH from 8.0 to 4.4 was accomplished by introducing 0.30 M NaH_2PO_4 through a 250 ml constant-volume mixing chamber containing the starting buffer. When the limit of the gradient was reached, salt concentration was further increased by introducing a 5% NaCl solution through the mixing chamber. Collection of the effluent in 3 ml fractions at a flow rate of 9 ml/hr was begun immediately after introduction of the sample. Optical densities of the fractions at 280 mμ were determined in a Beckman DU spectrophotometer and pH determinations were made in a Cambridge electron ray pH meter; fractionation was at 4°C.

Immunologic study. Large volumes of effluent were pooled and dried by lyophilization, reconstituted in water, and dialyzed against 0.85% NaCl. The concentrated pools were examined for antigenic activity by double

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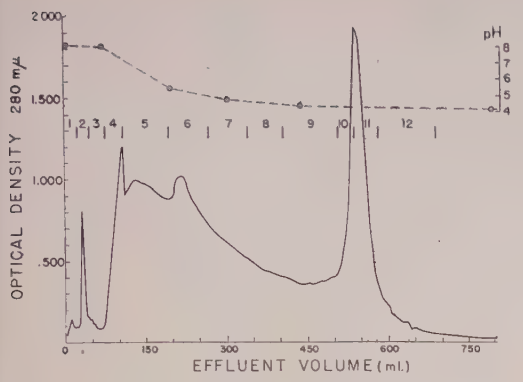

FIG. 1. Chromatogram of partially purified histoplasmin on DEAE-cellulose. Solid line represents optical density of column effluent at 280 mμ. Broken line and scale at right denote pH changes. Vertical lines demarcate pools of effluent.

diffusion in agar, using for antibody the serum from a person wth proven histoplasmosis.

Results. Fig. 1 shows the chromatogram obtained by plotting optical densities at 280 mμ against effluent volume. The pooled fractions are indicated in the figure by vertical lines, while the horizontal broken line and the scale at the right show pH changes. Pools 1 through 4 failed to exhibit antigenic activity when examined by the Ouchterlony technic. Pools 5, 7, and 8 each developed a single line while pool 6 and the crude antigen developed

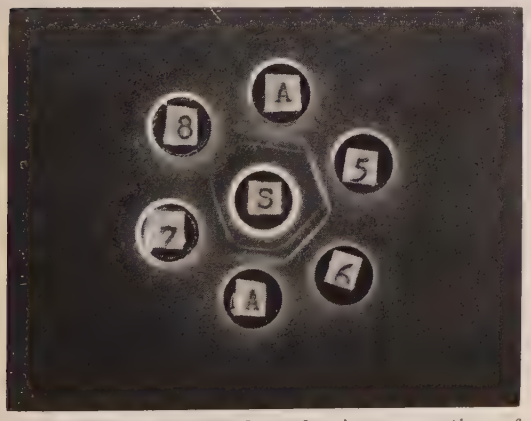

FIG. 2. Ouchterlony plate showing separation of "h" and "m" antigens. S: antiserum from patient with proven histoplasmosis; A: crude antigen; 5, 6, 7, and 8: pools of effluent from chromatographic column.

† We are greatly indebted to Dr. D. C. Heiner, who kindly identified the 2 lines developed with this antiserum as being due to the "h" and "m" antibodies.

2 lines corresponding to the "h" and "m" antigens of Heiner (Fig. 2).† One of these made identity with the line from pool 5 ("m" antigen), while the other made identity with those from pools 7 and 8 (the "h" antigen). No antigens were detectable in the remaining pools (9 through 12) by this very sensitive test.

Discussion. As demonstrated above, the "h" and "m" antigens of *H. capsulatum* can be separated from each other by column chromatography on DEAE-cellulose. In addition, a considerable degree of purification has been accomplished by removal of some 75-80% of inert material. Neither the precise degree of purity of these preparations nor the extent of contamination, if any, with other antigens has been determined. The crude mycelial phase histoplasmin used in this work, however, has produced no bands other than "h" and "m" when tested aganst several hundred sera from patients with culturally proven histoplasmosis or with strong presumptive evidence of the disease. It appears to be devoid of the other antigens reported by Heiner(4). The isolated fractions produced only one band when tested against several sera from culturally proven cases of histoplasmosis, or from those with strong presumptive evidence of the disease. Further purification and characterization of these preparations, as well as results of their use in immunologic tests, will be reported later.

Summary. "h" and "m" antigens apparently specific for detection of histoplasmosis have been separated from crude histoplasmin and purified by means of column chromatography on DEAE-cellulose. A gradient elution scheme with decreasing pH and increasing molarity of phosphate was used.

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Stability of Injected Vitamin B_{12}-Co^{60} and Vitamin B_{12} Content of Dog Liver. (26038)

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Interpretations of biological studies with cobalt-labeled Vit. B_{12} customarily assume that the administered tracer remains in tissue as the intact vitamin. The need for experimental support for this assumption has been recognized by many investigators(1,2). Scattered information indicates that orally administered Vit. B_{12}-Co^{60} exists in the human, at least initially and in part, as undegraded vitamin(3). A similar conclusion was drawn from examination of urine of rats receiving comparatively large doses of the labeled vitamin subcutaneously(4). The stability of Vit. B_{12} injected at physiological doses and retained by tissue for prolonged periods has not received much attention. Reports of examination of a single dog liver receiving 8.25 μg of labeled vitamin would indicate that Vit. B_{12} is stored at least partially (up to $\simeq$ 64%) intact(2,5). The present communication presents results of an investigation of livers from 5 dogs receiving 3.6-14 μg (specific activity $\simeq$ 1 mc/mg) of Vit. B_{12}-Co^{60} intravenously. The dogs were sacrificed 5-71 days following injection, and tissues were immediately stored in the frozen state for 10 months until assayed for cyanocobalamin and for non-cyano forms by isotope dilution methods (6). By this means it was possible to demonstrate that the administered labeled vitamin retained in the livers was present as intact cobalamin and probably as cyanocobalamin. It was further shown by a reverse isotope dilution experiment(6), that amount of free cobalt produced was negligible. Finally, an estimate of total cobalamin concentration was made by direct isotope dilution assay (6,7,8,9,10) employing the *in situ* radioactive cobalamin-Co^{60} as tracer, and in certain instances, with added Vit. B_{12}-Co^{57}. The isotope dilution method for assaying biological and pharmaceutical preparations for total cobalamins is amenable to modification by omission of initial cyanide treatment, so as to yield a value for cyanocobalamin alone.

Experimental. Animals. One of the dogs involved (282) was the subject of an extensive study of biliary excretion of Vit. B_{12}-Co^{60} reported recently(1) by several of the present authors. Three of the remaining animals were employed for an irradiation study. Dog 274 was normal. Relevant information such as weights, vitamin doses, and vitamin tissue content is compiled in Table I. The radioactive vitamin constituted but a negligible part of total Vit. B_{12} activity of these livers.

Procedures. In the reverse isotope dilution assay for total cobalamin, 50 g of tissue was homogenized with water in a Waring blendor to a total volume of $\simeq$ 250 ml, and $\simeq$ 10 mg (known amount) of crystalline cyanocobalamin added as carrier. To $\simeq$ 125 ml were added $\simeq$ 600 mg of $NaNO_2$ and 250 mg of KCN, and the whole heated to boiling for an hour before proceeding with the isolation procedure(9,10) for purified Vit. B_{12}. In addition to the standard procedure a zinc defecation step(7) and more rigorous acid washing were introduced. When only cyanocobalamin was sought, initial KCN was omitted. Carrier was of course omitted in direct isotope dilution assays with *in situ* Vit. B_{12}-Co^{60} or with added Vit. B_{12}-Co^{57}. In these analyses 100-200 g samples of tissue were employed, and both nitrite and cyanide were added. Cobalt chloride was employed as carrier (10 mg Co^{++}) for the reverse dilution assay for freely exchangeable or ionic cobalt. Approximately 50 g of homogenized liver containing the carrier cobalt was equilibrated by long standing at room temperature and by heating for an hour. The homogenate was then filtered, and ionic cobalt was extracted as the thiocyanato complex by means of secondary amyl alcohol. Cobalt was determined colorimetrically by the thiocyanate method(11). Cyanocobalamin content of carrier solutions and of mixed iso-

TABLE I. Experimental Animals (Males)—IV Administration.

Dog	Wt (lb)	Vit. B_{12}-Co^{60} dose (μg)	Days post-inj. at sacrifice	Liver wt (g)	Vit. B_{12} in liver: μg vit. B_{12}-Co^{60}	Vit. B_{12} in liver: Total μg	Comments
282	52	3.6	5	906	.7	400	Biliary fistula experiment
274	32	14.3	25	650	3.4	400	Normal
276	22	3.6	70	304	.3	73	Received 890 R x-radiation unilaterally
328	35	3.6	71	420	.4	135	
320	31	3.6	71	400	.4	84	Bilateral irradiation, total dose 290 R

topic products isolated from liver was determined spectrophotometrically. Radioactivity was measured by gamma ray scintillation counting in a well-type NaI.T1 crystal. When Co^{57} and Co^{60} labeled tracers were both present, gamma scintillation spectrometry was employed to distinguish between modifications.

Confirmatory evidence of the identity of recovered radioactivity with cyanocobalamin was obtained in all cases. In 2 instances, identification was made by chromatography on paper(12) using 0.01% KCN in secondary butanol (water saturated) as a developing solvent. This correspondence was established in all cases by determining radioactivity and color (where possible) distribution coefficients between the immiscible solvent pair water-butanol.

Results and discussion. Table II is a summary of results of reverse isotope dilution assays of 5 livers. This table contains percent recovery of radioactivity as cyanocobalamin alone and as total cobalamins. In all cases but the liver of dog #276, both total and cyanocobalamin account for approximately 100% of total radioactivity suggesting that the administered Vit. B_{12}-Co^{60} which resides in these tissues is still present as intact cyanocobalamin. Paper chromatography of total cobalamin isolate from a sample of liver from dog #274 revealed but one component in which radioactivity and Vit. B_{12} color (visible and spectrophotometric assay) coincided. Final confirmation of the cobalamin nature of the radioactive isolate is seen in the values of distribution coefficients (Columns 4 and 5, Table II) determined colorimetrically and radiometrically. The essential equality of these 2 values (in a given isolate) supports the common origin of color and radioactivity, *i.e.*, cyanocobalamin. It was also noted, in agreement with an earlier report(2), that radioactivity was distributed uniformly throughout the liver since different samples of a given tissue exhibited comparable (within 10%) cpm/g.

TABLE II. Recovery of Injected Cyanocobalamin-Co^{60} from Dog Livers.

Dog	% Co^{60} recovered: As cyanocobalamin	% Co^{60} recovered: As total cobalamins	Dist. coeff.: R'act.	Dist. coeff.: Color
282	99	103	1.18	1.21
	100	100	1.25	1.21
274	98	98	1.23	1.18
	92	104	1.26	1.16
276	56	97	1.34	1.20
	66	98	—	—
328	96	95	1.26	1.24
320	94	95	1.25	1.21

Reverse dilution assay of one liver sample (dog #274) for free or readily exchangeable cobalt yielded a value of $\simeq$ 1.0% of total radioactivity. Further purification would probably lower this value still further. Obviously, appreciable degradation of Vit. B_{12} to low molecular weight (ionic) cobalt compounds does not occur.

In view of the apparent stability of injected Vit. B_{12}-Co^{60} retained by liver, this endogenous labeled vitamin was utilized as an *in situ* tracer for the isotope dilution assay of liver for total cobalamin content. Isolation was performed as described in the reverse dilution procedure for total cobalamins. No attempt was made to distinguish between cyano and non-cyanocobalamins. Values of total cobalamins are listed in Table III. Radioactiv-

TABLE III. Vitamin B_{12} Content of Dog Livers.

Dog	Time lapse, mo*	μg vit. B_{12}/g Isotope Co^{60}	Dilution Co^{57}	$K_{dist.}$ (Co^{60})
282	10	.44	—	1.21
	22	.10	.08	
		.06	.07	
274	10	.62	—	—
	22	.14	.14	
276	10	.24	—	1.21
328	10	.32	—	1.28†
	22	.28	.24	
320	10	.21	—	1.29
Avg of initial assays		.36		

* Between sacrifice of animal and sampling for assay.

† Color distribution coefficient was 1.21.

ity (Co^{60}) distribution coefficients (K_{dist}) reported in column 5 again support the supposition that the isolate is authentic Vit. B_{12}. Furthermore, a paper chromatogram after addition of carrier vitamin to the isolate from dog #274 consisted of a single radioactive spot which coincided with the location of visible color. Using initial (10 months in frozen state) assays, the isotope dilution method gives an average cobalamin concentration of 0.36 μg/g of liver. This is of the order of Vit. B_{12} concentrations reported for mammalian livers(13,14). The drop in successive assay values for the dog livers is attributed to a gradual decrease in tissue cobalamin content, despite storage in the frozen condition during the over-all lapsed time of ≃ 2 years. A similar loss in activity was observed by microbiological assay(15) with *L. leichmannii*. This may be due to microbial infestation while specimens were thawed for sampling. That retained Vit. B_{12}-Co^{60} constitutes an authentic tracer is demonstrated by the agreement with values (Table III) obtained from dilution experiments with Vit. B_{12}-Co^{57} in the cases of livers 282, 274 and 328.

No attempt was made, in performing liver assays by direct isotope dilution, to differentiate between cyano- and non-cyano forms, only total cobalamins being determined. Vit. B_{12} activity appears to exist in tissue, at least in part, as hydroxocobalamin or other non-cyano analogues(16,17). This is in contrast to the observation that injected cyanocobalamin is retained by liver essentially unchanged. It is unlikely that digestion with nitrite alone would generate sufficient cyanide ion to convert non-cyanocobalamin-Co^{60} to the cyano form. It is more likely that the vitamin B_{12} present in tissue, being derived from exogenous sources, is ingested as a non-cyano analogue, whereas injected cyanocobalamin is retained intact. That residence times in tissue between 5 and 71 days yielded the same complete cyano-cobalamin-Co^{60} recoveries indicate the great stability of this compound in tissue.

Summary. Vit. B_{12}-Co^{60} injected intravenously into dogs is retained by dog livers as an intact cobalamin, probably as cyanocobalamin. This was demonstrated by the method of reverse isotope dilution. Direct isotope dilution indicates an average total cobalamin concentration of 0.36 μg/g of dog liver. The authenticity of retained Vit. B_{12}-Co^{60}, introduced by injection, as an isotope dilution tracer was demonstrated by simultaneous analysis with Vit. B_{12}-Co^{57}. Degradation of Vit. B_{12} to ionic cobalt is negligible.

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Vitamin B_{12} Activity of 5,6-Dimethylbenzimidazolylcobamide Coenzyme for the Chick. (26039)

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The isolation and properties of 5,6-dimethylbenzimidazolylcobamide coenzyme (DBC coenzyme), which is structurally similar to Vit. B_{12}, have been described(1,2). This coenzyme is required for conversion of glutamate to β-methylaspartate by cell-free extracts of *Clostridium tetanomorphum*(2), and also for isomerization of methylmalonyl-CoA to succinyl-CoA(3-5). The DBC coenzyme has activity similar to that of Vit. B_{12} for supporting growth of *Ochromonas malhamensis* and the Vit. B_{12}-requiring mutant of *Escherichia coli*(2). Since presence of the coenzyme has been demonstrated in rabbit liver, it is reasonable that this compound may also be a coenzyme form of Vit. B_{12} for higher animals. In the present study, activity of the DBC coenzyme was compared with that of Vit. B_{12} for supporting growth of young chicks. A second parameter used for assessing DBC coenzyme activity was suppression of formiminoglutamic acid (FGA) excretion by Vit. B_{12}-deficient chicks. This metabolite of histidine, which is excreted in negligible quantities by normal animals, was found to be excreted in large amounts in urine of Vit. B_{12}-deficient rats(6). The same finding has been observed in Vit. B_{12}-deficient chicks (unpublished data).

Methods. Day-old female New Hampshire chicks were distributed into groups of 8 chicks each and maintained for a period of 4 weeks in electrically heated batteries with screen wire floors. The chicks were weighed at weekly intervals. The diet was fed *ad libitum.*

Diet C62, which was used throughout this study, had the same composition as Diet C47 (7) except that 200 g of the glucose was replaced by an equal weight of hydrogenated vegetable oil (Crisco) in each kg of diet. Also, the Salts A used in Diet C47 was replaced by an equal quantity of Salts N(8). Vit. B_{12} was incorporated in this diet at levels shown in Table I.

Since the DBC coenzyme is destroyed by light, it could not be given in the diet. The chicks that received either Vit. B_{12} or the DBC coenzyme by injection received no Vit. B_{12} in the diet. In each case, aqueous solutions of supplements were injected intraperitoneally. The chicks fed a suboptimal level of Vit. B_{12}, 5 μg/kg of diet, served as the reference group in determining quantity of either Vit. B_{12} or DBC coenzyme to be injected. Food intake of this group was measured 3 times weekly and the groups receiving the 5 μg level of "supplement" by injection received exactly the same amount of Vit. B_{12} or DBC coenzyme as Vit. B_{12} eaten by the reference group. For convenience, in tables and text reference is made to weights of Vit. B_{12} and

TABLE I. Growth of Chicks Receiving Graded Levels of Vit. B_{12} and DBC Coenzyme.

Supplement (μg)*	Vit. B_{12} fed		Vit. B_{12} inj.		DBC coenz. inj.		Light-treat. DBC coenz. inj.	
	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)	No. exp.†	4 wk wt ± S.E. (g)
0	6	188 ± 7.8	—	—	—	—	—	—
1	—	—	2	213 ±11.0	2	247 ±12.4	1	254 ±11.9
2.5	2	216 ±14.4	5	250 ± 9.0	5	250 ± 8.1	—	—
5	6	260 ± 8.3	6	278 ± 8.1	4	267 ±11.0	4	276 ± 7.2
100	6	306 ± 7.6	—	—	—	—	—	—

* Quantity of vit. B_{12} fed/kg of diet. Amount of vit. B_{12} inj. was based on food intake of chicks receiving 5 μg vit. B_{12}/kg diet. DBC coenzyme was inj. in amounts equimolar to indicated weights of vit. B_{12}. See *Methods* for details.

† 8 chicks/experimental group.

DBC coenzyme. This was strictly correct only for Vit. B_{12}; the indicated quantities of coenzyme were actually equimolar to Vit. B_{12}.

Crystalline DBC coenzyme, isolated from *Propionibacterium shermanii* ATCC 9614(2), was dissolved in water. Concentration of the coenzyme was calculated from its extinction coefficient at 522 mμ and 260 mμ(2). To avoid destruction by light, the DBC coenzyme solutions were stored in dark containers and all of the precautions described by Barker *et al.*(2) were observed. To provide an additional control, a solution of DBC coenzyme was inactivated by exposure for 1 hour to a 100 watt incandescent light bulb at a distance of 6 inches. The coenzyme solution was in an open beaker in an ice bath. Ultraviolet and visible absorption spectra following this treatment were similar to those of Vit. B_{12b} (aquocobalamin).

The chicks used for the FGA experiments were fed the Vit. B_{12}-free diet *ad libitum* throughout. One per cent L-histidine HCl was added to the diet to increase FGA excretion. The chicks received distilled water during collection periods. At 24 days of age the chicks were transferred from the battery to individual stainless steel cages provided with extra heat from light bulbs.

The excreta collected prior to 30 days of age served as base-line control values. Between 31 and 52 days of age some of the chicks in both experiments were injected daily with 1 μg Vit. B_{12} or an equimolar amount of the DBC coenzyme. In the second experiment *all* chicks were injected with 100 μg Vit. B_{12}/day between 53 and 59 days of age. Excretion of FGA was determined on each day's samples for several days preceding and following a change in supplementation; at other times 24 hour samples were analyzed at 2 to 3 day intervals.

The excreta were collected daily on sheets of polyethylene placed under the cages. These samples were stored at –10°C until time for assay. Each sample was suspended in distilled water in a Waring blendor, frozen (at least overnight), thawed, and centrifuged. The supernatant liquid was decolorized with 10 mg activated charcoal/ml (Darco KB) and filtered through Whatman No. 12 fluted filter paper. The pH of the extract was kept below pH 6 to avoid destruction of FGA. Concentration of FGA in these extracts was determined colorimetrically using the alkaline ferricyanide-nitroprusside reaction(9). The samples were read in an Evelyn colorimeter and a standard curve was included in each assay. A composite of extracts containing high amounts of FGA served as the standard. FGA content of the standard was determined microbiologically(10).

Results. The mean 4-week weights of chicks receiving Vit. B_{12} or DBC coenzyme are presented in Table I. Weights of chicks that received no Vit. B_{12} averaged 188 g, whereas those that received 100 μg Vit. B_{12}/kg of diet averaged 306 g. Addition of either 2.5 or 5 μg Vit. B_{12} to the diet resulted in intermediate weight gains. Vit. B_{12} was utilized to the same extent whether injected or fed. Similarly, the DBC coenzyme supported growth equal to that of Vit. B_{12}. The higher mean weight of chicks injected with the 1 μg level of DBC coenzyme as compared to that with Vit. B_{12}

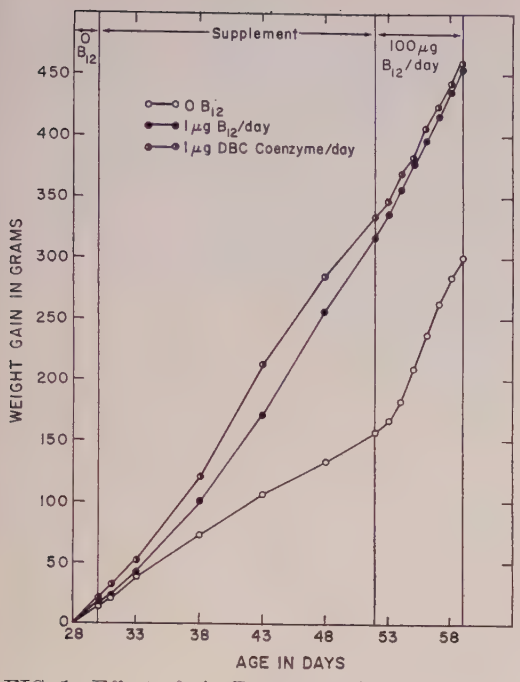

FIG. 1. Effect of vit. B_{12} and DBC coenzyme upon growth of 4-wk-old vit. B_{12}-deficient chicks (Exp. 2 of Table II).

was not statistically significant. Further, mean weights of chicks receiving 1 μg DBC coenzyme or 1 μg light-treated coenzyme were essentially the same. Therefore, the activities of Vit. B_{12} and DBC coenzyme are considered to be the same. These experiments offer no clue as to the physiologically active form of Vit. B_{12} in the chick, *i.e.*, Vit. B_{12} might be converted to the coenzyme form or the reverse might also occur.

From preliminary experiments, it was found that the high excretion rate of FGA in the Vit. B_{12}-deficient chick decreased somewhat slowly upon Vit. B_{12} supplementation. If the DBC coenzyme were the form of Vit. B_{12} active in producing a drop in FGA excretion, it seemed reasonable that the coenzyme might cause a more rapid and perhaps greater drop than was seen with Vit. B_{12}. The chicks in the early studies received 100 μg Vit. B_{12}/kg of diet and their daily food consumption was such that they received about 2 to 3 μg Vit. B_{12}/day. For the present experiments the level of supplementation was reduced to 1 μg/day in an effort to accentuate any possible difference between Vit. B_{12} and DBC coenzyme in their effect upon FGA excretion.

Growth rates of the chicks in the second FGA experiment are presented in Fig. 1. Chicks receiving Vit. B_{12} or DBC coenzyme grew at equal rates, as would be expected from the above growth studies with day-old chicks. Supplementation with 100 μg Vit. B_{12}/day markedly increased rate of gain of the Vit. B_{12}-deficient chicks; however, it did not further accelerate rate of gain of the previously supplemented chicks.

Data on FGA excretion are presented in Table II. All chicks at beginning of experiment were excreting large amounts of FGA, a little over 40 μmoles/24 hours/100 g body weight in the first experiment and somewhat higher amounts in the second experiment. This confirms in the chick the effects observed in Vit. B_{12}-deficient rats by Silverman and

TABLE II. Effect of Vit. B_{12} and DBC Coenzyme upon Excretion of Formiminoglutamic Acid by the Chick (μmoles FGA/Day/100 g Body Wt).

Group No.	Supplement* / Age of chicks, days	None 29–30	1 μg/day 31	32	33–34	51–52	100 μg/day 53	54	55–59
	Exp. 1								
1	None	44	38	40	32	36	—	—	—
2	B_{12}	44	46	20	14	14	—	—	
3	DBC coenz.	42	21	10	14	16			
	Exp. 2								
1	None	82	52	63	55	74	27	26	18
2	B_{12}	127	76	46	37	22	16	15	14
3	DBC coenz.	74	47	31	30	10	5	4	5

* Vit. B_{12} deficient chicks were inj. daily with 1 μg vit. B_{12} or an equimolar quantity of DBC coenzyme (Groups 2 and 3) at age of 31-52 days. On the 53rd day every chick in all groups received 100 μg vit. B_{12}/day. Diet C62 was supplemented with 1% L-histidine HCl. Five chicks/exp. group.

Pitney(6). The unsupplemented chickens continued to excrete large amounts of FGA. Both Vit. B_{12} and the DBC coenzyme caused a marked reduction in FGA excretion, which was down to minimal levels by the end of 2 days. The daily 100 μg Vit. B_{12} supplement, which was given to *all* chicks in the second experiment, caused a very marked and immediate drop in FGA excretion of Vit. B_{12}-deficient chicks that had not been previously supplemented. This very high supplement of Vit. B_{12} also caused a further reduction of FGA excretion by the 2 previously supplemented groups. Thus, by both parameters, growth and reduction of FGA excretion, Vit. B_{12} and DBC coenzyme had similar activities.

Summary. Vit. B_{12} and the 5,6-dimethylbenzimidazolylcobamide coenzyme were equally active in (1) supporting growth of chicks up to 4 weeks of age and in (2) overcoming the impaired metabolism of formiminoglutamic acid that occurs in Vit. B_{12}-deficient chicks.

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Fragility and Extractable Collagen in the Lathyritic Chick Embryo. An Assay for Lathyrogenic Agents.* (26040)

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The lathyrogenic substances, β-aminopropionitrile and semicarbazide, when introduced into the chick embryo induce a loosening of the intermolecular structure within collagen fibrils allowing them to dissolve in cold neutral saline(1). This intrafibrillar alteration is associated with marked increase in fragility of the entire organism. Both phenomena are demonstrable between one and 3 hours following a single injection and increase steadily for at least 72 hours. Both were also shown to be dosage dependent. Analytical studies(1) plus histologic and electron microscope examination of thin sections of normal and lathyritic skin before and after extraction(2) led to the conclusion that insoluble fibrils, formed prior to administration of the agent, were rendered soluble in cold saline. Skin, bone, aorta and tendon were all involved.

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It is the primary purpose of this report to establish the basis for a bio-assay for lathyrogenic activity utilizing induced chick embryo fragility and extractability of collagen.

Procedures. Groups of 20 to 30 fertilized eggs of the white Leghorn variety were injected at 14 days of incubation with a variety of agents dissolved in 0.2 ml of sterile saline. Application was made through a pinhole onto the chorioallantoic membrane as described previously(1). After 48 hours of further incubation embryo fragility was measured, and tissues prepared for extraction.

Mortality rate for all doses was no higher than that for controls, about 10%.

Fragility was measured by determining the weight required to detach the head from the body within a period of 10 to 100 seconds. Three to 5 embryos from groups of 20 were used in preliminary estimates of the proper weight. A more reproducible but somewhat longer procedure involved a continuous load increase to the breaking point, thus eliminating the time factor. This is accomplished by flow of water at a fixed rate into a cellophane bag suspended from the head of the embryo. A useful fragility index was found to be the ratio of the breaking weight for controls to that for experimental embryos.

A biochemical index of activity was obtained by measurement of viscosity and hydroxyproline content of cold saline extracts of minced bones pooled from 10 to 20 treated embryos. Tibiae and femora were stripped of muscle, tendon and periosteum, minced with scissors and extracted in 3 volumes (v/w) of 1 M cold saline containing phosphate 0.02 ionic strength pH 7.6 at 5°C for 24 hours with shaking. The extracts were centrifuged at 100,000 g in the Model L Spinco preparative ultracentrifuge for 1 hour. Supernatant solutions were passed through fine sintered glass filters and viscosity was measured in Ostwald viscosimeters with a flow time of 60 seconds at 5°C. Each extract was analyzed for hydroxyproline and in one experiment (Table I) hexosamine and tyrosine were also determined as described previously(1). Standard curves were obtained using 1, 3, 5, 10, 20 and 30 mg of BAPN fumarate. As a routine only 3 and 10 mg points were used.

TABLE I. Dosage Dependence of Bone Extract Properties and Embryo Fragility.

Dose	Fragility index	η rel	μg/ml of extract		
			Hypro	Hexosamine	Tyrosine
Saline	1.0	1.5	17.0	74.6	240
BAPN fumarate					
2 mg	1.9	3.3	46.0	91.0	314
5	2.5	8.4	122.5	76.3	308
10	5.0	20.0	285.0	80.4	312
20	7.7	23.2	385.0	87.0	310
30	9.1	22.0	390.0	78.8	300

The following substances§ were tested in this series: water, β-aminopropionitrile fumarate (BAPN), aminoacetonitrile, methylene aminoacetonitrile, methylaminoacetylnitrile, semicarbazide, β,β'-iminodipropionitrile, β-alanine, β-mercaptoethylamine, cystamine, taurine, methylamine and cyanoacetic acid.

Iproniazid phosphate (1-isonicotinyl 2-isopropyl hydrazine),‖ an inhibitor of monoamine oxidase, said to enhance lathyrogenic activity of BAPN in the turkey poult(3) was applied as described above in doses of 10^{-2}, 10^{-4}, and 10^{-6} moles, alone and with 3 mg of BAPN.

Results. Data from a representative experiment are shown in Table I. Dose relationships for fragility index and extract viscosity and hydroxyproline were nearly linear between 1 and 10 mg of BAPN and parallel each other. Maximum effect was obtained at about 20 mg. Tyrosine and hexosamine content of the extracts were elevated to about the same degree at all doses.

Table II reports the effectiveness of a number of analogues and substances of known lathyrogenic activity in the rat, as compared to β-aminopropionitrile in equimolar amounts.

Iproniazid alone produced none of the stigmata of lathyrism in the chick embryo, although it increased mortality in doses of 10^{-2} moles. It did not significantly enhance the effectiveness of BAPN. Maximum dose used was much greater than those said to completely inhibit liver monoamine oxidase in the rat and guinea pig(4).

§ Courtesy of Abbott Laboratories.

‖ Courtesy of Roche, Inc.

TABLE II. Relative Activity of Compounds.

Agent	Molar equivalents of BAPN in terms of: Fragility	Viscosity	Hydroxyproline
$NH_2CH_2CH_2CN$ (β-aminopropionitrile)	1.0	1.0	1.0
NH_2CH_2CN (aminoacetonitrile)	2.5	2.5	4.0
$NH \cdot CONHNH_2$ (semicarbazide)	.3	.3	.3
$NH_2 \cdot SC \cdot NH \cdot NH_2$ (thiosemicarbazide)*			
$CH_2{=}NCH_2CN$ (methylene aminoacetonitrile)	2.0	2.5	4.0
$CH_3NHCOCN$ (methylaminoacetylnitrile)	0	0	0
$NH(CH_2CH_2CN)_2$ (β,β'-iminodipropionitrile)	0	0	0
$NH_2CH_2CH_2SH$ (β-mercaptoethylamine)	0	0	0
$(NH_2CH_2 \cdot CH_2S)_2$ (Cystamine)	0	0	0
$NH_2CH_2CH_2SO_2H$ (taurine)	0	0	0
$NH_2CH_2CH_2COOH$ (β-alanine)	0	0	0
$HOOC_2CH_2CN$ (cyanoacetic acid)	0	0	0
NH_2CH_3 (methylamine)	0	0	0
$NaOOC \cdot CH{=}CH \cdot COONa$ (sodium fumarate)	0	0	0

* Thiosemicarbazide was highly active in a dose of 25 mg but was not assayed quantitatively.

Discussion. Embryo fragility and appearance of extractable collagen were always associated following application of lathyrogenic compounds. Dosage dependence for both phenomena were sufficiently close to linear for the range 1 to 10 mg of BAPN to provide a useful quantitative measure for comparison of the effectiveness of various agents.

Analyses for hexosamine, a measure of aminosugar containing compounds such as glycoproteins and mucopolysaccharides, and for tyrosine, a rough measure of non-collagenous proteins indicated no correlation with dosage in either case. However, actual isolation of the large sugar-containing substances is required for any satisfactory evaluation of changes in relative amounts or properties of such molecules.

In practice an unknown compound may be assayed by comparing its effectiveness to that of two different doses of BAPN fumarate, for example 3 and 10 mg (using the same batch of eggs), the comparison being made on a molar equivalent basis.

Among the groups of agents examined in this study, only 5, known to be lathyrogenic in the rat, proved to be effective, namely β-aminopropionitrile, aminoacetonitrile, methylene aminoacetonitrile, semicarbazide, and thiosemicarbazide.§ β - mercaptoethylamine and cystamine are said by Dasler *et al.*(5) to produce lathyrism in rats, a conclusion disputed by Ponsetti *et al.*(6). These compounds proved to be inactive in the chick embryo in the doses used (9 and 18 mg, respectively). The reported enhancement of lathyrogenic activity by iproniazid(5), a blocking agent for monoamine oxidase, also could not be confirmed in this system.

The series of agents reported is still too small to permit assessment of important common denominators.

We suggest that physical chemical changes such as increased tissue fragility and extractability of collagen are more sensitive and measurable indices of lathyrogenic activity than are morphologic changes, that they represent a phase of the process closer to the biochemical defect, and that their measurement provides a useful tool for deeper exploration of pathogenetic mechanisms.

Summary. A quantitative assay for lathyrogenic agents utilizing induced fragility and extractability of collagen from bones of the chick embryo is reported. Both parameters are dosage dependent and parallel each other. The method has merits of ease, rapidity, reproducibility, and single dose requirement. Fifteen compounds consisting of 5 known lathyrogens and a number of analogues were assayed. Iproniazid phosphate had no enhancing effect on lathyrogenic activity. No correlation between dose and extractable hexosamine and tyrosine were found.

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Comparative Metabolic Studies of H^3 and C^{14} Labeled Stearic Acid.* (26041)

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With the introduction of liquid scintillation spectrometers, beta-emitting tracers began appearing more frequently in studies using radioisotopes. Tritiated compounds have been thought advantageous because of ease in preparation, high specific activity, low cost, and relative safety. However, we have often considered whether H^3 might also be fraught with properties that do not lend it to accurate tracing of specifically labeled fatty acids. With these thoughts in mind, the following investigation was undertaken to compare metabolism of H^3 and C^{14} labeled stearic acid.

Materials and method. Tissue absorption studies. Approximately 0.6 mg of stearic-1-C^{14} and 0.15 mg of stearic-9, 10-H^3 (5-10 μc) were mixed in 1 cc of corn oil and fed by means of stomach tube into male Sprague-Dawley rats weighing 300-450 g. They were maintained on a diet of Purina Fox Chow before and during experiments. Animals were decapitated at intervals of 15, 30, 45, 60, 90, 120, 180, and 240 minutes. Livers and epididymal fat pads were excised and homogenized in methanol or immediately frozen at –15°C. Tissue lipids were extracted with chloroform-methanol as described by Van Handel and Zilversmit(1). Five milliliter aliquots of the extracts were then evaporated and placed in counting solvent, consisting of 0.4% PPO† and 0.01% POPOP‡ in reagent grade toluene. Counts were made in a Packard Tri-Carb Liquid Scintillation C-14 Spectrometer. Simultaneous equations were then used to determine the separate values contributed by each of the 2 labels when both were counted simultaneously. Counting at HVT 5, window 10-80, efficiencies of around 50% for C^{14} and 0.3% for H^3 were obtained; while at HVT 9, window 10-50, efficiencies of around 11% for C^{14} and 15% for H^3 were obtained. Thirty-two animals were studied in this manner. *Lipid fraction studies.* In these analyses the dose was raised to 50 μc per type of stearic acid so as to increase sample specific activity. Aliquots analyzed by the silicic acid columns were reduced to 0.0125 cc for fat and 0.025 cc for liver compared to the 5 cc aliquots used in the absorption studies. Animals were decapitated 4 hours post injection, liver and fat pad were weighed, then immediately placed in the homogenizer. Chloroform-methanol extracts were further processed to remove protein contamination by the method suggested by Enteman(2), using a flash evaporator at 60°C, successive chloroform-methanol extractions, and filtration through fat-free filter paper. The lipid obtained was then fractionated on silicic acid columns according to the method of Hanahan (3). To check the manner in which differently labeled fatty acids were handled by the silicic acid columns, we put known concentrations of stearic-1-C^{14} and stearic-9, 10-H^3 on the columns and then carried out the usual elution procedure.

Results. Liver and epididymal fat pad absorption. Variation when comparing H^3 to C^{14} in specific activity of the labels in each tissue is apparent. Variations of similar magnitude were found in the counts of the same type labeled stearate in different animals killed at the same time (Table I). On some

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† PPO or 2, 5-diphenyloxazole.

‡ POPOP or p-bis (2-(5-phenyloxazole))-benzene from Pilot Chemicals, Inc., Watertown, Mass.

TABLE I. H^3 and C^{14} Stearic Acid Absorption Studies.*

Min.	% H^3 dose/g tissue, mean & stand. dev.		% C^{14} dose/g tissue, mean & stand. dev.	
Fat pad	%			
15	.818	.80	1.09	1.27
30	2.25	2.32	2.18	1.47
45	9.0	1.65	7.2	3.88
60	10.9	6.82	13.5	11.9
90	38.4	14.3	46.8	33.2
120	26.0	23.5	36.8	29.2
180	105.5	75.3	114.8	90.7
240	94.1	61.4	114.0	83.0
Liver				
15	2.0	.61	1.5	.23
30	6.1	3.24	7.6	4.4
45	29.7	8.8	27.8	11.9
60	58.4	27.6	43.9	20.6
90	198.1	157.0	134.4	66.3
120	192.6	335.0	172.4	123.0
180	365.2	232.0	320.0	197.6
240	405.8	107.6	410.9	254.0

* Each measurement made in 4 animals.

occasions C^{14} deposition seemed greater than H^3. But all these mentioned variations were not consistent nor statistically significant ($p>0.5$). Uptake of each label in the tissues examined seemed most rapid in 1½ hours. Also, specific activity of liver lipid was greater than that of the adipose tissue.

Lipid fractionation studies. The 4 fractions separated by silicic acid chromatography were: (a) cholesterol esters, (b) triglycerides and free fatty acids, (c) free cholesterol, monoglycerides, diglycerides, (d) phospholipids. Results in this part of the investigation showed a tendency for C^{14} and H^3 stearic acids in the fat pads to go to fraction b, less to fraction c, and a negligible amount to fraction d. In liver the labels were found to be concentrated in fraction d with a small but significant amount in fractions b and c (Table II). More H^3 was incorporated into the lipid fractions with the exception of liver phospholipid.

Table III summarizes our findings when stock C^{14} and H^{13} stearic acid were run through the silicic acid column. This points up a consistent and fairly sizable contamination of the tritiated fatty acid into the free cholesterol, monoglyceride and diglyceride fraction, and a lesser contamination in the cholesterol ester fraction. No such contamination was found in the case of C^{14}.

Discussion. Since its introduction, tritium has seemed attractive as a label for use in tracer studies. It has been used widely in industry with apparent success and more recently in many biochemical studies(4). However, the question arises as to whether H^3 would stick firmly to a fatty acid vehicle—not be liberated and/or exchanged freely with non-radioactive H + during any significant chemical manipulation. It was hoped that this study would help elucidate some of the tracer properties of H^3 for fatty acids. In looking for a vehicle fatty acid we decided upon stearic acid in that the work of Glascock and Reinius(6) indicated the H^3-stearic bond was stable. Our absorption studies in-

TABLE III. Recovery Studies.

Fraction	Stearic-1-C^{14}	Stearic-9, 10-H^3
	% activity	
a	.38	1.41
b	97.30	89.60
c	1.78	8.55
d	.54	.49

TABLE II. Tissue Lipid Fractionation Studies. % administered dose/100 g tissue.

	H^3					C^{14}				
	Exp.					Exp.				
Fat pad	1	2	3	4	5	1	2	3	4	5
	(%)									
a	.0	.8	.0	.0	.0	.0	1.2	.0	.0	.0
b	4.3	5.9	7.1	12.0	5.7	4.4	7.1	10.6	17.9	4.7
c	.55		.81	1.1	1.2	.65		9.4	1.9	.32
d	.21	.23	.01	1.2	.8	1.1	1.9	2.0	1.6	2.2
Liver										
a	.15	.51	.18	.17	.27	.16	.9	.45	.56	.38
b	2.3	7.8	5.7	5.3	4.4	3.8	6.5	8.1	9.4	6.5
c	.28	.75	.93	.65	.69	.42	.84	1.4	1.5	.66
d	13.8	33.0	29.0	28.0	31.0	12.0	22.8	32	28	15.0

dicate no G. I. alteration of the H^3-stearic acid compared to C^{14}-stearic acid.

During chloroform-methanol lipid extraction, the possibility was considered that H^3 might exchange with H_2O washings. This possibility was checked by testing portions of the non-lipid water-methanol fractions for radioactivity; none was found. From this it would appear that the label deposited in both liver and epididymal fat pad remained in the lipid as extracted.

Due to previously demonstrated aberrations in phospholipid fractions during fatty acid studies(1,7) in which a fatty acid molecule was altered by a label, the remainder of this work dealt with comparisons of H^3 and C^{14} within the lipid fractions as separated by silicic acid columns. Variation was most evident in the fat pad, especially for both fraction c and fraction d. Finding the label in fraction c, especially for the fat pad, was of interest. It has been shown that fat tissue produces little cholesterol(8,9). This then represented incorporation of the labels into monoglycerides and diglycerides, deposition of liver cholesterol in the fat pad, or was of some significance in generation of cholesterol. Because of this finding plus the fact that H^3 seemed to show this phenomena more than C^{14}, we considered the possibility that this resulted from alterations to the H^3 label brought on by the silicic acid columns. 8.6% of the stock H^3 labeled stearic acid turned up in fraction c (Table III) when processed through the column. Here, then, was the first glaring example of apparent inaccuracy in use of the H^3 labeled stearic acid. Even accounting for H^3 stearic acid contamination, the count in fraction c is impressive. According to the studies in rat liver of Dittmer and Hanahan(3) using stearic-1-C^{14}, stearic acid has a propensity to incorporate into both phospholipid and esterified cholesterol. What we observed, then, may be an extension of this phenomenon to fat tissue, may represent some form of cholesterol transport, or may indicate incorporation of H^3 into monoglycerides and diglycerides. Further investigation already under way seems to indicate the last possibility.

Summary. The stability of orally administered stearic-9, 10-H^3 appears comparable to that of stearic-1-C^{14} in its incorporation into lipid in rat liver and epididymal fat pad. However, column fractionation of tissue lipids and stock labeled fatty acids suggests a difference in behavior of stearic acid labeled in these 2 ways.

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Growth of Feline Viral Rhinotracheitis Virus in Cultures of Feline Renal Cells. (26042)

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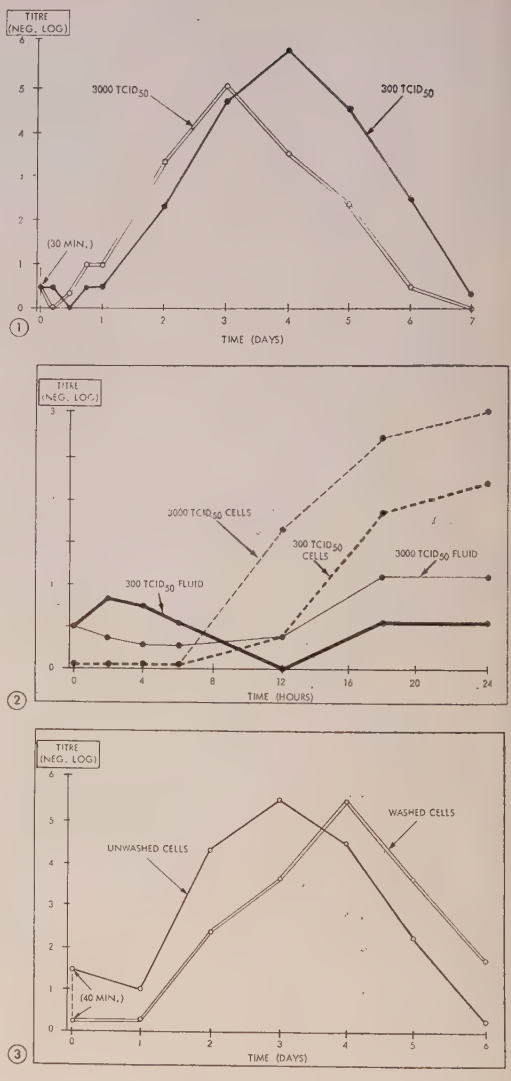
The isolation of a virus which induces rhinotracheitis in cats and produces a cytopathic change in cultures of feline renal cells has been reported(1,2). This report deals with the growth of feline viral rhinotracheitis (FVR) virus in cultures of feline renal cells and the

correlation between release of infective virus and appearance of inclusion bodies.

Materials and methods. Methods for preparation and maintenance of feline renal cell cultures have been described(2). The maintenance medium at time of virus inoculation was 0.5% lactalbumin hydrolysate in Hanks balanced salt solution fortified with 3% lamb serum. The 5th tissue culture passage of the original isolate that had a $TCID_{50}$ of 10^{-5}/0.1 ml was used throughout. One group of culture tubes was inoculated with 0.1 ml of virus containing a calculated 300 $TCID_{50}$ dose. A second group received 0.1 ml of virus containing a calculated 3,000 $TCID_{50}$ dose. At selected time intervals following inoculation, the fluid from 3 tubes at each dosage level was harvested and pooled. The cells were removed from the glass surface with a rubber "policeman" and washed 3 times in maintenance medium. The fluid and cell harvests were stored at –20°C until assayed. At the time the fluid and cell harvests were made, collodion sections were prepared from replicate tubes and stained with hematoxylin and eosin for cytologic studies. For virus assay, fluid and cell samples were thawed at room temperature. The cells were disrupted by addition of 0.2-mm glass beads and shaken on the Mickle tissue disintegrator for 3 minutes. For titrating the cellular and extracellular virus, serial 10-fold dilutions were prepared, and 0.1 ml of each dilution was inoculated into each of 4 feline renal culture tubes, which were observed for 6 days. Virus titres were calculated by the method of Reed and Muench and are expressed as the negative logarithm per 0.1 ml of that dilution which caused cytopathic change in 50% of the cultures. Fluid and cells collected during the first 18 hours were also tested for viral content in the undiluted form.

In another experiment, two 32-oz. prescription bottles containing a monolayer of feline renal cells were inoculated with 500,000 $TCID_{50}$ doses of virus in a total volume of 50 ml of maintenance medium to determine(1) when maximum titre would occur if large quantities of virus were used, and (2) the effect on the growth curve of washing the cell



layer. The fluid from 1 bottle was removed after 30 minutes of incubation, and the monolayer was washed 3 times with maintenance medium. The original volume was replaced with the latter medium. The first fluid sample was harvested from both bottles 40 minutes following inoculation with the virus. One milliliter of fluid was then removed at 24-hour intervals from each bottle for a period of 6 days and stored at –20°C until assayed. These samples were titrated as described above.

Throughout the study all cultures were incubated at 37°C. A simultaneous titration of the virus pool was performed each time the

tubes and bottles were inoculated and $TCID_{50}$ dose calculated.

Results. The growth curves shown in Fig. 1 represent results obtained in cultures inoculated with 300 $TCID_{50}$ or 3,000 $TCID_{50}$ doses of virus. The titre at 30 min for the 300 $TCID_{50}$ curve was less than 1 log. At 12 hr no virus could be demonstrated, but newly released virus was found at 18 hours. This steadily increased to a maximum virus titre of $10^{-5.6}$ on the 4th day. The logarithmic decrease following the peak titre corresponded to the release of cells from the glass surface.

The level of measurable virus at 30 minutes with the 3,000 $TCID_{50}$ level was not significantly different from the 300 $TCID_{50}$ dose curve. At 6 hours there was no detectable fluid virus, but the 12-hr sample indicated early multiplication. The titre between 18 and 24 hours was 10^{-1} before ascending rapidly to a peak level of 10^{-5} on the 3rd day. The logarithmic decrease was earlier with the 3,000 $TCID_{50}$ dose than with the lower dose. About 3 days after the maximum titre in both curves, the titre was almost undetectable.

The amount of cellular virus demonstrated during the first 24 hours is shown with the extracellular (fluid) virus in Fig. 2. Cellular virus infective for tissue culture was not detected during the first 6 hours but was present at 12 hours. With the 300 $TCID_{50}$ inoculum a small amount of virus was demonstrated intracellularly at 12 hours, but none was detected in the fluids at this time. Virus content of the cells which had received the higher dose was more than 1 log higher than that in the fluid at 12 hours. Beyond 12 hours until approximately at 48 hours, cellular virus concentration remained considerably higher than extracellular virus. The extracellular virus then exceeded the cellular virus by about 1 log in titre.

Results of replicate growth curves demonstrating extracellular virus for the 2 levels of inoculum during the first 12 hours, sampled at 30, 50, 70, 90, and 110 minutes and at 2-hour intervals thereafter, were in agreement with the curves shown in Fig. 2. Although there were differences in amount of detectable virus for each curve during the first 12 hours, it was always less than 0.5 log.

The effect upon the growth curve of washing the monolayer 30 minutes following inoculation is shown in Fig. 3. The fluid titre 40 minutes following virus inoculation from the culture bottle in which the monolayer was not washed was $10^{-1.5}$. This decreased to 10^{-1} by the 1st day. After the 1st day, virus content increased rapidly and yielded a maximum titre of $10^{-5.5}$ on the 3rd day. Virus titre decreased steadily until it was $<10^{-1}$ by the 6th day. The amount of fluid virus in the washed culture bottle during the first 24 hours remained constant at $<10^{-1}$. Quantities of virus released into the fluid increased after the 1st day at a slower rate than in the unwashed bottle and attained a maximum titre of $10^{-5.5}$ on the 4th day. Rate of decrease of virus titre from the 4th day to the 6th day was similar to that in the unwashed bottle.

Nuclear changes suggestive of virus activity were observed in the cells inoculated with 3,000 $TCID_{50}$ doses at 18 hours, but were not observed in the cell cultures inoculated with 300 $TCID_{50}$. Well-formed intranuclear inclusion bodies were demonstrated in stained preparations of feline renal cell cultures 24 hours following inoculation of 300 and 3,000 $TCID_{50}$ doses of virus.

Discussion. When cultures of feline renal cells were infected with 2 different dosages of FVR virus, a characteristic viral growth curve was obtained; *i.e.,* following a brief latent period when little or no detectable virus was present, virus content increased to a maximum yield and then declined. The only significant difference demonstrated by comparing the 2 inocula was in time; namely, a shorter latent period, more rapid rise to approximately equal titre, and an earlier degeneration of the cell layer at the higher dosage level. The decrease in detectable virus which preceded the release of new extracellular virus can be explained as the period of attachment or absorption of virus to the cells and/or a period of eclipse when no virus was detectable in the cells. It is apparent that the virus was in a state which cannot be detected by infectivity tests for the period between 6 and 12 hours postinoculation.

Nuclear changes with well-formed inclusions were observed about the time that extra-

cellular virus was demonstrated. We did not, however, observe nuclear alteration at the time new virus was detectable within the cells, as reported with polio virus(3). Mature inclusion bodies have been demonstrated concomitantly with release of extracellular virus in growth studies of canine hepatitis virus (4,5).

Unpublished results in our laboratory show that the FVR virus is antigenically unrelated to the kidney cell degenerating virus (KCD) (6), which also was obtained from a cat. In addition to antigenic differences, these 2 viruses can be differentiated on the basis of their respective growth pattern and cytopathic changes in cultures of feline renal cells. FVR virus multiplied at a slower rate than the KCD virus, which, when an inoculum of 1,000 $TCID_{50}$ doses was used, reached its peak titre by the 21st hour. The main cytopathic differences between FVR virus and KCD were the presence of intranuclear inclusions and the longer time required for cytopathic change to appear in infected cultures with the FVR virus.

Summary. The growth pattern of feline viral rhinotracheitis virus in cultures of feline renal cells was investigated. Following an initial period of 18 to 24 hours during which little or no virus could be detected, virus concentration increased steadily until a maximum titre of $10^{5.5}$ was reached between the 3rd and 4th days. Intranuclear inclusion bodies were demonstrated in infected cells at the time of release of new extracellular virus.

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Studies on Tetracycline-resistant *Escherichia coli* Strains and a Growth-requirement for l-Histidine. (26043)

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In contrast to its tetracycline-sensitive parent, it was found that a laboratory-developed, tetracycline-resistant *E. coli* strain would not grow in Witkin M-9 synthetic medium(1). Additional studies indicated that the tetracycline-resistant strain had a specific growth requirement for l-histidine. Attempts were made to determine whether or not a metabolic relationship existed between histidine and tetracycline resistance in clinically-isolated, tetracycline-resistant *E. coli* strains.

Methods and discussion. The parent *E. coli* has been maintained a number of years in this laboratory by regular transfer in brain heart infusion agar. The tetracycline-resistant strain was selected by a serial transfer procedure in brain heart infusion broth(2). In the current studies, the parent *E. coli* was maintained on M-9 synthetic agar medium. Surface growth was washed off, sedimented by centrifugation, and washed 3 times with 10 ml aliquots of saline. The tetracycline-resistant strain was maintained on brain heart infusion agar. Such cells were washed as above with 3 separate aliquots of 10 ml sterile saline.

Growth was determined as percent of incident light transmitted in the Evelyn photoelectric colorimeter. This approximated 50% transmittance (660 mu filter) for the parent *E. coli* in M-9 medium after 18 hours incubation at 37°C. In contrast, the laboratory-developed, tetracycline-resistant *E. coli* failed to show any indications of growth in this medium even after 48 hours incubation at 37°C.

In an effort to supply a necessary growth factor, a variety of amino acids, vitamins, indole, and purine bases (at 10 μg/ml) in

TABLE I. Effect of Adjuvants on Growth of *Escherichia coli* (Tetracycline-Resistant) in Witkin M-9 Medium.

Adjuvant group*	Composition of group	Growth of *E. coli*, as % light transmittance, %
I	l-Leucine dl-Threonine l-Isoleucine l-Histidine l-Methionine	54
II	Cytosine l-Valine l-Phenylalanine l-Tryptophane	99 (no growth)
III	Indol Guanine l-Arginine l-Alanine	99 (no growth)
IV	Thiamin Riboflavin Pantothenate Folic acid Biotin	99 (no growth)
Individual components of Group I	l-Leucine	99
	dl-Threonine	99
	l-Isoleucine	99
	l-Histidine	50-60
	l-Methionine	99
Controls		
Parent *E. coli* (tetracycline-sensitive) in M-9 medium		52
E. coli (tetracycline-resistant) in M-9 medium		99

* 10 μg/ml of each component adjuvant.

groups of 4 or 5, were added to the M-9 medium. These data are presented in Table I. Only the group composed of l-leucine, dl-threonine, l-isoleucine, l-histidine, and l-methionine permitted growth, which was 54% light transmittance after 18 hours at 37°C. The 3 remaining groups of adjuvants failed to permit growth even after 48 hours incubation. Of the individual amino acids in Group 1, l-histidine was the only one which permitted growth to 50-60% light transmittance within 18 hours. It appeared obvious, therefore, that this tetracycline-resistant strain of *E. coli* selected by a laboratory-procedure was a histidine-requiring auxotroph. Such auxotrophic mutants of *E. coli* are not uncommon (3).

A number of diverse compounds have been shown to play a role in synthesis and/or degradation of histidine in microorganisms (4,5). A number of these, *i.e.*, urocanic acid, imidazole, 5-imidazole analogs, l-histidinol, and l-histidinal at concentrations of 1, 10, and 100 μg/ml could not replace the specfic and definite requirement for l-histidine in the tetracycline-resistant *E. coli* (Table II).

It was of interest to determine whether or not l-histidine and several of the compounds mentioned above would antagonize the activity of tetracycline against the parent and the tetracycline-resistant *E. coli* strains. None of the compounds had any demonstrable effect on tetracycline activity against the parent *E. coli* (Table III). Although this strain is resistant to 100 or >100 μg/ml of tetracycline when tested in natural medium (brain heart infusion broth), MIC (minimum inhibitory concentraton) is less when tested in synthetic medium, *i.e.*, 12.5-25 μg/ml.

To determine the relative occurrence of the requirement for l-histidine among other strains of *E. coli* resistant to tetracycline, a number were obtained from clinical sources in two widely-separated parts of the United States. All were found to have minimum inhibitory concentrations of 100->100 μg/ml

TABLE II. Specificity of l-Histidine as Growth-Requirement for Tetracycline-Resistant *E. coli* in M-9 Medium.

Organism	Adjuvant	Conc., μg/ml	Growth of *E. coli*, as % light transmittance, %
E. coli (tetracycline-resistant)	None		99 (no growth)
Idem	l-Histidine	10	52
"	Urocanic acid	100	99
		10	"
		1	"
"	Imidazole	100	99
	2-(p-Nitrobenzyl-mercapto) imidazole	100	"
	2-(Phenylthio-carboxy) imidazoline	100	"
	2-(m-Nitro-henyl-thiocarboxy) imidazoline	100	"
	2-(p-Chlorophenylthiocarboxy) imidazoline	100	"
	Benzimidazole	100	"
"	l-Histidinal	100	"
"	l-Histidinol	100	"

TABLE III. Failure of Adjuvants to Alter Minimum Inhibitory Concentration of Tetracycline against *E. coli* Strains in M-9 Medium.

Organism	Medium	μg/ml of adjuvant		MIC of tetracycline
E. coli (tetracycline-sensitive)	M-9			1.56
	"	l-Histidine,	10	1.56
E. coli (tetracycline-resistant)	" + 10 μg/ml l-histidine			12.5
Idem	*Idem*	Urocanic acid,	100	12.5
			10	12.5
			1	12.5
"	"	Histidinol,	100	12.5
"	"	Histidinal,	100	12.5
"	"	l-Histidine,	100	12.5
			200	12.5
			400	12.5
"	"	Imidazole,	100	12.5

for tetracycline as determined by the 2-fold serial dilution technic in brain heart infusion broth. All the 20 strains received were able to grow in the synthetic medium approximately as well as the parent laboratory strain. Therefore, none of these tetracycline-resistant strains, which were developed naturally *in vivo*, had a requirement for l-histidine.

Summary. 1. A tetracycline-resistant *E.* coli, which had been selected by a laboratory technic, was found to have a specific requirement for l-histidine. 2. Urocanic acid, imidazole and 5 analogs, l-histidinol, and l-histidinal were unable to substitute for l-histidine, nor were they able to antagonize the activity of tetracycline against the parent *E. coli* (tetracycline-sensitive) in synthetic medium. 3. Twenty *E. coli* strains, all tetracycline-resistant and isolated from clinical sources were obtained. None had the specific requirement for l-histidine.

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On the Mechanism of Decrease of Aldosterone Secretion in the Dog. (26044)

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Previous experiments(1) have suggested that the integrity of the vagus nerves is necessary for the decrease in secretion of aldosterone following release of constriction of the thoracic inferior vena cava, but not for the increase in secretion of aldosterone following caval constriction. Further, vagotomy alone has no effect on aldosterone secretion in the otherwise intact animal. This suggests that the vagal pathway is strictly an inhibitory one, and that release of this inhibition does not result in increased secretion of aldosterone. Other experiments(2,3) have indicated that stretch of the right atrium results in diminished secretion of aldosterone. These experiments suggest a possible mechanism for activation of the vagal pathway.

However, several questions concerning the function of the vagus nerve in control of secretion of aldosterone have remained unan-

swered. First, it is possible that efferent rather than afferent fibers in the vagi may be important. Second, it appeared not unlikely that the vagal system, if it were an afferent pathway, might also be able to lead to increased secretion of aldosterone if other pathways ordinarily involved in increase in secretion of aldosterone were inactivated. Recently, it has been shown(4) that the integrity of the baroceptor nerves arising from thyro-carotid arterial junctions is necessary for the increase in aldosterone secretion which normally follows caval(1) or carotid(4) constriction. It was not clear whether vagotomy would lead to increased aldosterone secretion in a dog with thyrocarotid areas denervated. In the third place, it has been shown(5) that bilateral constriction of the common carotid arteries produces an increase in right atrial pulse pressure without changing atrial mean pressure. Caval constriction, on the other hand, produces a decrease in atrial mean and pulse pressures(6). Thus, release of carotid constriction should not provide a means of activating atrial receptors similar to that present following release of caval constriction. Consequently, if aldosterone secretion decreases following release of carotid constriction, it should do so more slowly than it does following release of caval constriction. The present experiments were designed to answer these questions.

Methods. Experiments were performed on healthy mongrel dogs anesthetized with pentobarbital. Adrenal venous blood was collected intermittently by the method of Hume and Nelson(7). All collections were of one half hour's duration. Adrenal venous aldosterone was measured by Mills' modification (1,4) of the method of Neher and Wettstein (8). This method was further modified in some experiments by substitution of tritium-labelled aldosterone for C^{14} cortisone as the tracer. This added the theoretical advantage that the tracer used for correction of losses ran with the unknown aldosterone in all 3 chromatographic systems, rather than only the first two. Caval constriction(1), vagotomy(1) and carotid constriction(4) were all performed as reported previously. All blood lost through collection or incidentally was replaced by homologous transfusion. Arterial, venous and right atrial pressures were measured and recorded continuously.

Four experiments were performed on intact dogs with inflatable cuffs around the thoracic inferior vena cava. Following a control collection of adrenal venous blood, the cuff was inflated, and a second collection begun after one half hour. Following this collection, the cuff was deflated and a third collection made after one hour. The cuff was then reinflated and a fourth collection was made after one half hour. At the end of the second collection, each dog was injected with 2 mg of atropine intravenously (external jugular) and 2 mg intramuscularly.

Four experiments were performed on dogs in which both thyro-carotid arterial junctions had been denervated one to 2 weeks previously. The vagi were isolated low in the neck before experiment was begun. Following a control collection of adrenal venous blood, both vagi were sectioned. Further collections of adrenal venous blood were obtained at intervals from 30 to 240 minutes following vagotomy.

Five experiments were performed on intact dogs with silk loops passed about both carotid arteries. The design was similar to that in the dogs with caval constriction, except that carotid rather than caval constriction was used, and no atropine was given.

Results. The results of caval constriction and release in dogs given large doses of atropine are shown in Fig. 1A and Table I. Release of caval constriction in presence of massive amounts of atropine resulted in decreases of aldosterone secretion (mean −5.53 μg/hr ± 1.68 SEM) not different from those seen in normal dogs (mean −5.14 ± 1.32) and in marked contrast to the effect of release of

TABLE I. Effect of Caval Constriction and Release on Aldosterone Secretion (μg/Hr) in Normal Dogs Given Atropine (2 mg i.v. and 2 mg i.m.) at End of Second Collection.

Control	Constriction	Release	Constriction
1.8	5.2	2.5	3.8
1.5	8.2	2.3	6.2
14.0	18.0	8.6	3.4
5.0	11.4	7.3	12.0

Effect of release: mean fall 5.53 ± 1.68 S.E.M.

TABLE II. Effect of Vagotomy on Aldosterone Secretion (μg/Hr) in Dogs with Previous Denervation of the Thyro-carotid Arterial Junctions. Vagotomy performed at end of first collection.

	Time after section (min.)			
Control	30-60	120-150	180-210	210-240
1.5	1.0	1.1	1.6	
1.2	.6	1.4	1.4	
<1.4*	<1.4	<1.4	1.5	
4.5	4.6	4.1	—	<3.8

* When < is shown, no aldosterone was detected in the sample. The figure shown is calculated from sensitivity of the method, recovery rate, and dilution of sample. See ref.(4).

caval constriction in vagotomized dogs (mean −0.79 ± 1.86)(1). It is evident that the effect of vagotomy is not reproduced by atropine. Atropinization also did not prevent the rise in aldosterone secretion following caval constriction in 3 of the 4 dogs. The situation was complicated in the fourth dog by the onset of atrial fibrillation following injection of atropine, and reversion to normal rhythm with the second caval constriction. Thus, atrial pressure rose markedly in this animal after caval constriction.

The results of vagotomy in dogs with bilateral denervation of the thyro-carotid arterial junctions are shown in Fig. 1B and Table II. As is the case with intact dogs(1), vagotomy produces no change in aldosterone secretion.

The results of release of carotid constriction in normal dogs are shown in Table III and summarized and compared with the results of release of caval constriction in Fig. 2. The effect of carotid release is significantly less than that of caval release.

Discussion. These results suggest that the mechanism leading to decreases in aldosterone secretion mediated by the vagus nerves operates over afferent pathways and is exclusively

TABLE III. Effect of Release of Carotid Constriction on Aldosterone Secretion (μg/Hr) in Normal Dogs. Constriction had been on for one hr, and all dogs showed increase in aldosterone secretion above control levels.

Constriction	Release	Δ
11.9	10.8	−1.1
9.9	10.5	+ .6
8.6	6.9	−1.7
20.4	17.8	−2.6
17.0	14.0	−3.0

Mean = −1.56 ± .72 (S.E.M.) t = 2.19 p >.05

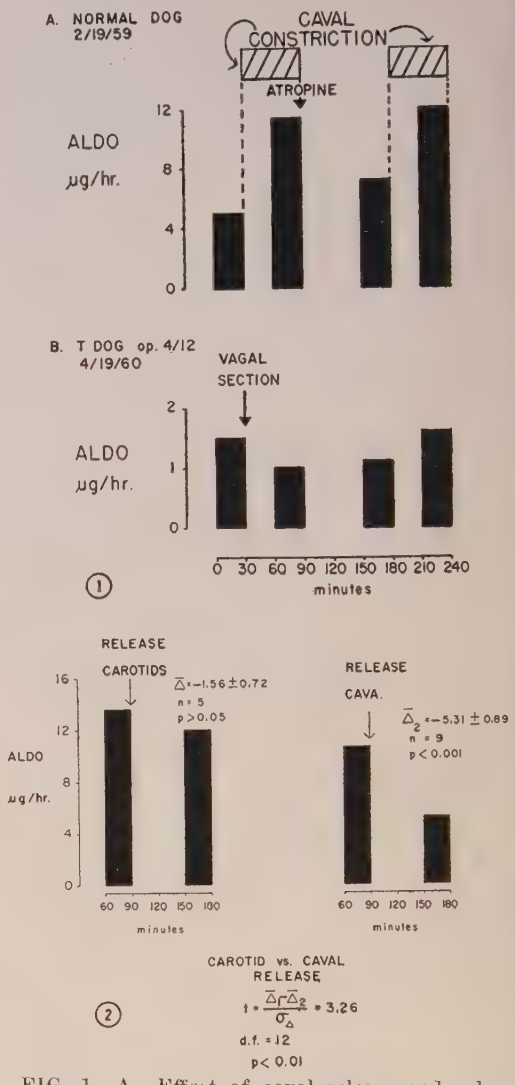

FIG. 1. A. Effect of caval release and subsequent caval constriction on aldosterone secretion in an atropinized dog. B. Effect of section of vagus nerves low in the neck upon aldosterone secretion in a dog one wk after thyrocarotid arterial junction denervation.

FIG. 2. Summary of experiments comparing aldosterone secretion after release of carotid arterial constriction with that following release of inferior vena cava constriction during acute experiments in the dog.

inhibitory in function. This situation, in which a reflex mechanism serves only to inhibit while a release of inhibition is not sufficient to stimulate, is unusual but is not unique in physiology. Alexander(9) has demonstrated a similar reflex, also utilizing afferent vagal pathways, in which venous tone

is diminished by increasing central venous pressure, but a fall in this pressure does not produce increased venous tone. This latter effect seems to be predominantly under the control of arterial baroceptors(10). The analogy between regulation of venous tone and that of aldosterone secretion is striking. It is of interest that an increase in secretion of aldosterone or in venous tone has the ultimate physiological effect of increasing the volume of blood available to the heart for perfusion of the arterial tree, and that both may be increased(11,12) in congestive heart failure. The difference between results of release of caval constriction, on the one hand, and of release of carotid constriction on the other, correlates with the different effects upon atrial pressure. This is consistent with the concept (1,2,3) that the vagal pathway is activated by atrial stretch, but does not exclude some other receptor area. Further support is provided by the fact that in dogs with prior denervation of the thyro-carotid arterial junctions, caval constriction produces no change, but carotid constriction produces a fall in aldosterone secretion(4). Thus, in the absence of the nerves arising from the thyro-carotid arterial junction, carotid constriction, which increases atrial pulse pressure(5) may activate the vagal mechanism. However, this evidence is not adequate to identify the hemodynamic parameter mediating decrease in aldosterone secretion or to distinguish whether type A or B atrial receptors may be involved(13).

Summary. Experiments were done in dogs to elucidate the mechanism by which the vagus nerves may influence aldosterone secretion. The results suggest that the vagus nerves form the afferent limb of a reflex activated by atrial stretch which may exert only an inhibitory effect upon aldosterone secretion. The role of efferent impulses was eliminated by atropinization, which did not prevent the fall of aldosterone secretion which follows caval release. The role of atrial stretch was suggested by the observation that caval release (which increased atrial pressure) lowered aldosterone secretion more than carotid release (which did not). The purely inhibitory role of the vagus was suggested by the finding that vagotomy did not stimulate aldosterone secretion even after denervation of the thyro-carotid arterial junctions.

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Hormonal Modification of Endotoxin Mortality in Mice.* (26045)

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Activation of the sympathetic nervous system, known to follow endotoxin administration, is held to be in part responsible for endotoxin death(1-4). Demonstrating activation Boquet(2) and Zweifach(3) have observed marked adrenergic vasoconstriction following intoxication, while Heiffer(4) has recorded adrenal depletion of the adrenalines in the rabbit. Antiadrenergic compounds, on the other hand, have been observed to protect several species against endotoxin death(5).

Several recent papers have examined perturbations of non-adrenergic amines following endotoxin administration. They describe an endotoxin-induced serotonin release from blood platelets, a phenomenon that eventuates in a transient increase in plasma serotonin followed by its disappearance from the blood during the shock state(6-8). In this work we are concerned with evaluating the possible influence of this phenomenon on endotoxin mortality. We approximate the condition by studying the effect of injected serotonin on endotoxin lethality, in mice. We attempt to modify this effect, in turn, by use of Compound F. Pertinent to this, glucocorticoids have been found to potentiate the eosinopenic response to serotonin(9).

An examination of thyroxine's modification of endotoxin mortality is appended to these studies. Our interest in such action stems from an observed increased sensitivity of thyroxine-treated animals to adrenaline(10-11). Similarity of subject matter to the main body of the experiment make this inclusion warranted.

These experiments were published some time ago in abstract form(12).

Methods. Swiss Webster and Carworth Farms mice were used, weighing between 22 and 26 g. Food and water were given *ad libitum*. Endotoxin was purified *E. coli* endotoxin (Difco): serotonin, the creatinine sulfate (Sigma): the Compound F, a suspension, Cortef, (Upjohn): and thyroxine, sodium l-thyroxine, (Nutritional Biochemicals).

Exp. 1. Four hundred and fifty Carworth Farms male mice were divided into 3 groups of 150 mice each. One group was injected subcutaneously with 0.8 mg/kg serotonin base, another with 1.6 mg/kg serotonin, and the third group with saline diluent. Thirty minutes later all animals were injected with 32 mg/kg endotoxin intraperitoneally. Mortality data were collected at 48 hours.

Exp. 2. Two hundred Carworth Farms female mice and 100 Swiss Webster female mice were divided into 2 groups each. One group of each strain was injected subcutaneously with 0.8 mg/kg serotonin in 0.1 cc saline and the other group was injected with the diluent alone. Thirty minutes later all animals were given endotoxin intraperitoneally. The Swiss Webster animals were given 24 mg/kg toxin and the Carworth Farms animals were given 32 mg/kg toxin, doses previously found to kill approximately ⅔ of the subjects in the respective strains. At 48 hours mortality data were recorded.

Exp. 3. Four hundred and thirty-five Carworth Farms male mice were divided into 4 groups of from 80 to 130 each. One group was injected subcutaneously with 1.2 mg/kg serotonin, and with 1.0 mg/kg Compound F subcutaneously at another site; another group was injected with serotonin alone and appropriate diluent; another was injected with Compound F alone and appropriate diluent; the fourth group was given 2 injections of diluent. Thirty minutes later all were injected with 32 mg/kg endotoxin intraperitoneally. Mortality data were collected at 48 hours.

Exp. 4. Swiss mice were divided into 4 groups of 70 each and were injected for 0, 1, 3, and 4 days with 50 γ l-thyroxine/day/

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TABLE I. Effect of Serotonin and Compound F on Endotoxin Toxicity in Mouse.

Exp.	Sex	Serotonin, mg/kg	Compound F suspension, mg/kg	Mortality	% mortality	% protection	P
1	♂	0	0	99/150	65		
	♂	.8	"	84/150	52	13	
	♂	1.6	"	64/148	42	23	.01
2	♀	0	"	102/150	67		
	♀	.8	"	48/150	31	36	.01
3	♂	0	"	72/111	64		
	♂	1.2	"	53/114	47	17	.01
	♂	"	1.0	34/130	30	34	.01
	♂	0	"	54/80	67	0	

adult mouse. At end of treatment period each group was given 8 mg/kg endotoxin intraperitoneally. Mortality data were collected at 48 hours.

Results. Table I illustrates serotonin protection against endotoxin mortality, and moderate enhancement of this effect by an otherwise silent dose of Compound F. The degree of protection in these experiments fell between 12 and 36 percent depending upon the dose of serotonin employed, the presence or absence of Compound F, and the sex of the test mice.

The effect of serotonin was moderate but significant at less than the 1 percent level. The increase of protection with F was likewise significant. As well, female mice were significantly more responsive to serotonin than males at the dose tested. Table II illustrates a thyroxine enhancement of endotoxin mortality that increases progressively with the treatment period. Through this influence an LD 11 is increased to an LD 91 after 4 days.

Discussion. Vulnerability to the lethal effects of endotoxin may be modified by the action of diverse circulating hormones. Spink has observed protection in the mouse exerted by glucocorticoids(13). Our studies reveal small amounts of serotonin to be protective in the mouse, and demonstrate potentiation of this effect by a quantity of Compound F suspension not protective in itself. Conversely, a progressive loss of resistance to endotoxin death is observed to develop during thyroxine treatment.

TABLE II. Effect of Thyroxine on Endotoxin Toxicity in Mouse.

Days Rx	Mortality	Mortality
0	8/70	11
1	24/70	34
3	37/71	52
4	64/70	91

As other antiadrenergic compounds have proved to exert some protection the effect of serotonin may be related to an expression of its antiadrenergic action, elaborated on and reported by us elsewhere(14). It is not inordinate to propose that the "anti-endotoxin" action of injected serotonin may be exerted by the serotonin normal to the animal.

Compound F suspension had no effect on endotoxin mortality by itself, a function of the low dose and poorly soluble form in which it was used by us. The same amount of F given as a soluble compound (Solu Cortef, Upjohn) protects against endotoxin death in our hands, conforming to the observations of Spink.

The thyroid principle triiodothyronine has recently been reported to enhance endotoxin mortality in mice by Melby and Spink(15). These workers attributed their results to the augmented oxygen requirement induced by thyroid compounds. With this we are in agreement and suggest as well the parallel contribution of a thyroid potentiation of adrenaline vasoconstriction, forcing a reduction of oxygen available to the peripheral tissues regardless of tissue requirements(10).

Summary. Serotonin reduces endotoxin mortality in mice. This effect is greater in females, and is potentiated by Compound F. Maintained Thyroxine treatment progressively increases toxicity of endotoxin in this species.

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Effect of Urease Injection on Body Weights of Growing Rats and Chicks.* (26046)

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Antibacterial agents, fed in trace amounts, enhance growth of birds and animals under a variety of environmental conditions(1-4). Some investigators have postulated that these agents suppress toxins produced by gastrointestinal bacteria. Ammonia has been suggested as one of the toxins whose production is inhibited(5-8). We have recently reported that addition of 100 ppm of 3 antimicrobial agents to a casein diet significantly decreased *in vivo* hydrolysis of C-14 urea by rats and reduced production of urease by gastrointestinal bacteria(9). Our preliminary investigations have shown growth stimulation in rats and chicks immunized with jackbean urease (10, 11). Urease stimulates production of antiurease in rabbits which on passive transfer to normal rabbits produces a drop in blood ammonia(12). In the experiments herein reported the effects of jackbean urease injections on growth of rats and chickens fed soybean diets of suboptimal nutrient content are described.

Materials and methods. Urease was crystallized from jackbean meal and recrystallized twice from ethyl alcohol by the method of Kirk and Sumner(12). Enzymic activity was determined and purity of the preparations was checked using paper chromatography. The enzyme dissolved in 0.85% NaCl was injected intraperitoneally into rats and subcutaneously into chickens. Injections were made during the first 4 weeks of each experiment which lasted 8 weeks. In the rat experiments, injections were made every other day whereas in the chick experiment, injections were made on the same 3 successive days each week for the first 4 weeks. Control subjects received 0.85% NaCl in equal volume to that received by those which were immunized. In rats, the starting doses were 10 units† per kg of body weight, progressing to 25 units per kg body weight. Each rat received a total of 30 units of urease. The chicks received initially doses of 0.1 unit per injection and progressed to a dose of 10 units

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† One unit of urease activity is that amount of urease required to liberate one mg of ammonia N in 5 min. in a phosphate buffer at pH 7.0 at 20°C.

TABLE I. Body Weights of Male Sprague-Dawley Rats and Cockerels Immunized with Jack-bean Urease. (No./group shown in parentheses.)§

	0-4 wk	4-8 wk	0-8 wk
Chicks			
Wt gain, g			
Control (20)	405 ±11.7 *	777 ±35.8	1182 ±38.2
Exp. (19)	405 ± 9.8	905 ±26.6 ‡	1310 ±26.0 ‡
Avg feed/g gain			
Control (20)	2.02 ± .25	2.42 ± .19	2.28 ± .17
Exp. (19)	1.90 ± .29	2.27 ± .05	2.16 ± .15
Rats—Exp. I			
Wt gain, g			
Control (10)	151.2 ± 6.0	97.0 ± 4.9	248.2 ± 7.1
Exp. (20)	154.0 ± 4.2	107.8 ± 3.9	261.8 ± 6.9
Feed/g gain			
Control (10)	2.73 ± .07	5.37 ± .29	3.72 ± .06
Exp. (20)	2.69 ± .04	4.71 ± .12†	3.51 ± .05†
Rats—Exp. II			
Wt gain, g			
Control (10)	151.4 ± 4.4	101.9 ± 2.8	253.3 ± 5.6
Exp. (20)	150.1 ± 2.7	110.1 ± 2.8	260.2 ± 4.7
Feed/g gain			
Control (10)	2.63 ± .04	5.01 ± .13	3.58 ± .04
Exp. (20)	2.57 ± .03	4.59 ± .09†	3.41 ± .03†

* Stand. error of mean. † Statistically significant at 5% level. ‡ Statistically significant at 1% level. § Avg initial wt, g, for control and experimental groups respectively were: Chicks 38.3 ± 1.0 and 38.2 ± 0.6. Rats—Exp. I 39.2 ± 1.3 and 40.6 ± 0.4; Exp. II 56.9 ± 1.2 and 57.1 ± 0.8.

per injection in the fourth week. Each bird received a total of 52.5 units of urease. All experiments were conducted in a temperature controlled laboratory. The rats were individually housed in wire bottom cages and the chicks were maintained in batteries in groups of 10. Feed and water were provided *ad libitum*. Feed intakes and body weights were determined weekly. The basal diet for chicks was compounded as follows: solvent extracted soybean meal (50% protein) 40.0; sucrose 41.8; fat (Crisco) 8; cellulose (Alphacel) 4.0; salt mixture (U.S.P. XIV) 4.0; and vitamin mixture in sucrose 2.2. The vitamin mixture per 100 lb. of diet contained Vit. A acetate, 900,000 units; Vit. D (Viosterol) 100,000 units; dl-alpha tocopherol (250 I.U./g) 5 g; ascorbic acid 45 g; inositol 5 g; choline chloride, 75 g; menadione 2.25 g; para-amino benzoic acid 5 g; niacin 5 g; riboflavin 1 g; pyridoxine-HCl 1 g; thiamine-HCl 1 g; calcium pantothenate 3 g; biotin 20 mg; folic acid 90 mg; and Vit. B_{12} 1.35 mg. For the rat experiments the soybean oil meal of the diet was decreased to 31.4% and sucrose was increased accordingly. Two experiments with weanling male Sprague-Dawley rats having an initial weight of 40-50 g were conducted. Chicks used for the study were day-old cockerels of the Vantress-Arbor Acre cross.

Results. Chicks receiving urease injections gained an average of 1310 g in 8 weeks as compared to controls which gained 1182 g (Table I). This difference of 128 g in favor of the immunized birds is statistically significant ($P < 0.05$) and was the result of increased growth during the 4-8 week period since weight gains during the 0-4 week period were the same. Efficiency of growth expressed as g of feed per g of gain was in favor of the immunized birds during the entire experiment, being 2.0 *vs.* 1.90 during 0-4 weeks and 2.42 *vs.* 2.27 during 4-8 weeks. As an average for the entire 8 week experiment, the immunized birds required 2.28 g of feed per g of gain and controls required 2.16 g. Contrary to the results with rats, as described below, we were unable to demonstrate to our satisfaction the presence of antibodies in sera of the immunized chickens. This is not suf-

ficient evidence, however, to prove that antibodies to urease were not responsible for the growth effects observed(13-15).

In 2 successive experiments with a total of 60 rats, a combined test of significance demonstrated at a 1% level of probability that rats receiving urease injections require less feed per g of weight gained. In each experiment the feed required per g of weight gain was about 10% less for urease-treated rats than for control rats when calculated over the 4-8 week period (4.71 g per g of gain *vs.* 5.37 g per g of gain in Exp. I and 4.59 *vs.* 5.01 in Exp. II). In each case this difference is statistically significant at the 5% level. The increase in efficiency of food conversion to weight gain in the last four weeks of both experiments was responsible for overall differences in efficiency of food conversion for the entire 8 week experimental period (3.51 g of feed per g of gain *versus* 3.71 in Exp. I and 3.41 *versus* 3.58 in Exp. II). These differences are significant at the 5% level. Average gains in body weight were in favor of the immunized rats. In Exp. I average gain during the 4-8 week or post injection period was 97.0 g for control animals and 107.8 g for experimental animals. Corresponding values for Exp. II were 101.9 and 110.1. There was appreciable variation from rat to rat and none of the weight gain differences were statistically significant. In both experiments with rats the difference in average weight gains between control and treated animals for 0-4 weeks is rather small compared to its standard error. Since the rats had initially been distributed between control and experimental groups so as to make average initial weights nearly identical, it was thought that this effect might represent a close dependence between weight gain and initial weight. Analysis of covariance, however, demonstrated dependence on initial weight to be negligible and the adjusted estimates differed only trivially from those not adjusted. The figures shown in Table I are not adjusted for initial weight. No differences were observed in gain or feed required per gram of gain in the 0-4 week or injection periods.

At sacrifice no gross differences were observed between the organs of the immunized rats and their controls. Weights of adrenals, liver, spleen, and kidneys were not different between control rats and immunized rats. Sera collected from immunized rats were found to contain as much as 2.5 units of antiurease‡ per ml. The antisera protected normal rats against fatal doses of urease and inhibited the ureolytic activity of a phosphate buffer extract of gastrointestinal contents from these animals. Before sacrifice 4 randomly selected animals of the control group and 8 of the immunized group were studied for *in vivo* C-14 urea metabolism after having food and water withheld for 16 hours. In 4 hours after injection of C-14 urea, immunized animals expired an average of 2.58% of the radioactivity as $C^{14}O_2$ and control animals expired 4.31%. The urea splitting activity of the gastrointestinal contents of immunized animals averaged 40% less than that of controls. These differences were statistically significant ($P<0.05$).

Discussion. In all of the above experiments growth effects were not observed during the 0-4 week or injection period suggesting that antibody production had not reached a sufficient level. Experiments with rabbits in our laboratory indicate that easily detectable antiurease titers in the sera following a weekly schedule of urease injections do not occur until the third to fourth weeks. The time lapse before growth effects are evident suggests that antibodies are necessary to produce the results observed. The immunized rats of Exp. II had less urea splitting activity in their gastrointestinal contents as measured by 2 methods. Based on these and previous findings, it is reasonable to postulate that growth effects of dietary antibiotics and urease immunity would be additive. Antibodies to urease suppress the enzyme which is formed and antimicrobial agents decrease its synthesis(9). Our experiments now in progress with chicks fed soybean diets support this hypothesis.

Summary. Experiments with 40-day-old chicks and 60 weanling Sprague Dawley rats were conducted. The experimental subjects were immunized with jackbean urease injected

‡ A unit of antiurease is that amount of antibody which will inhibit one unit of urease activity.

during the first 4 weeks of experiments lasting 8 weeks. During the 4-8 week or post injection period, the immunized chicks gained 905 g *vs.* 777 g for their controls and were more efficient in converting feed to weight gain. In 2 successive experiments immunized rats grew slightly faster and showed a difference in feed efficiency which was statistically significant. In both chicks and rats there were no differences in body weight during the 0-4 week injection period. Antibodies to urease were demonstrable in sera of immunized rats by *in vivo* methods with C-14 urea and by *in vitro* tests. Soybean diets were fed.

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Haptoglobin Hemoglobin Metabolism in Rabbits Studied with I^{131} and Fe^{59} Labelled Complexes.* (26047)

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Several studies(1-3) attempting to trace the fate of haptoglobin-hemoglobin (HpHb) in man have pointed to the complexity of the problem. They have shown that the complex disappears from the blood either in a linear or exponential manner with a half life of about 4 hours, and it has been suggested that the complex may be removed *in toto* by the reticuloendothelial system(1). Studies in man have been restricted to short periods of observation because of the small amounts of hemoglobin that can be injected safely. The present experiments with I^{131}-labelled haptoglobin-hemoglobin (I^{131}HpHb) and haptoglobin Fe^{59} labelled hemoglobin (HpHbFe^{59}) complexes were undertaken in rabbits to follow the metabolism of the complex over longer periods of time. Attempts were also made to evaluate more directly the role of the reticuloendothelial system in the metabolism of the complex.

Materials and methods. Concentration of haptoglobin in serum was determined by starch zone electrophoresis on blocks, 45 cm x 33 cm, using potato starch and barbital buffer pH 8.6 μ 0.05 at 400 volts for 15-18 hours(4). Increasing amounts of hemoglobin were added to 0.1 ml aliquots of serum, and the mixtures were introduced into 3 parallel rows of 15 slits. After electrophoresis, control sera containing hemoglobin in 3-fold excess of the combining capacity demonstrated

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2 red spots. Corresponding test segments were eluted with 2-3 ml of 0.15 N NaCl and tested with benzidine. Forty-five specimens were studied simultaneously and as little as 1 mg haptoglobin/100 ml serum could be detected. Hemoglobin binding capacity was expressed as the largest amount of hemoglobin bound by the more rapidly migrating fraction. a_2 globulins were isolated from non-hemolized type 1-1 human serum by starch zone electrophoresis(4). Iodination of a_2 globulins and rabbit serum albumin at levels of less than 1 atom of iodine/molecule of protein was performed by standard methods(5). Unbound iodide was removed by passage through an ion exchange resin. Following addition of hemoglobin C, the iodinated a_2 globulins were re-electrophoresed to effect separation of I^{131} HpHb from the remainder of the I^{131} a_2 globulins(4). While separation was relatively complete, it seems likely that the haptoglobin was contaminated with about 10% slowly migrating a_2 globulin.

Hemoglobin was prepared by the method of Drabkin(6) from thoroughly washed erythrocytes from mice, and humans homozygous for hemoglobin A or C. Fe^{59} labelled hemoglobin was prepared in a similar manner from erythrocytes of mice 48 hours after intraperitoneal injection of mouse plasma containing 100 to 130 μc Fe^{59} buffered to pH 5.6. Five to 20 mg of hemoglobin containing 2 to 15 μc of Fe^{59} were mixed with haptoglobin prior to injection into the rabbits. I^{131} labelled proteins were assayed in a well type scintillation counter with a sensitivity for I^{131} of 9.05 x 10^5 c/min/μc above a background of 180 c/min. I^{131} and Fe^{59} combinations were assayed in a pulse height analyser. The base line and channel width were adjusted to settings such that Fe^{59} was counted free of I^{131} and a 15% correction for Fe^{59} was made at the I^{131} peak.

Study design. Tracer studies were performed in 13 rabbits weighing 2-5 kg. Since concentration of plasma haptoglobin was low (ranging from 5-30 mg/100 ml in 20 animals), non-radioactive haptoglobin was injected in the form of human serum, or whole a_2 globulins, to raise plasma concentration of the complex to approximately 25-50 mg/100

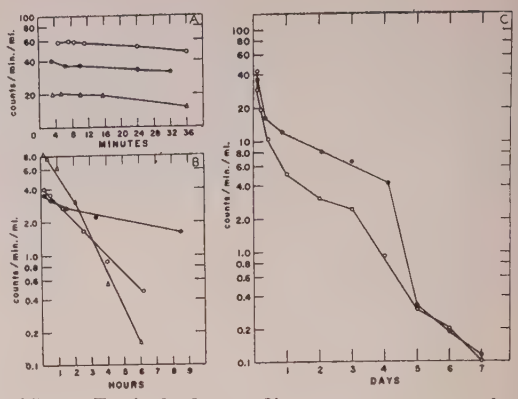

FIG. 1. Typical plasma disappearance curves for I^{131} HpHb (○), I^{131} a_2 globulins (●) and HpHb-Fe^{59} (△). While an initial rapid decrease in plasma concentrations was not observed (1A) plasma concentrations of I^{131} HpHb and HpHb-Fe^{59} decreased exponentially for 6-8 hr following inj. (1B). Three to 4 days following inj. plasma concentration of iodinated proteins fell sharply (1C, see text). I^{131} HpHb and I^{131} a_2 globulin plasma concentrations are expressed as counts/min./ml × 10^{-3} and HpHb-Fe^{59} as counts/min./ml × 10^{-2}.

ml. Two to 25 mg HpHb labelled with either 30-100 μc of I^{131} (8 studies) or 5-10 μc Fe^{59} (5 studies) were injected into an ear vein with sufficient hemoglobin to saturate the plasma haptoglobin. Heparinized blood was obtained at 2-5 minute intervals from the ear opposite to the side of injection for 30 minutes and at greater intervals for 4-6 days. Radioactivity was determined in plasma and following precipitation with cold 20% trichloracetic acid (TCA). In specimens containing Fe^{59}, precipitation with TCA was performed after hydrolysis with 2 N HCl to release Fe^{59} bound to transferrin(7).

The effect of HpHb on rate of clearance of carbon was studied in 200-300 g rats according to the method of Biozzi, Benacerraf and co-workers(8). Ten to 12 mg carbon particles per 100 g body weight were injected intravenously. Following the appearance of a steady rate of removal of carbon, 15-35 mg of HpHb were injected intravenously, and the effect on rate of carbon clearance was noted. The role of the liver in removing HpHb from the plasma was studied in 2 rabbits, by injecting a mixture of albumin I^{131} and HpHb Fe^{59} into the portal vein and drawing blood immediately from the hepatic vein. The ratio Fe^{59}/I^{131} was determined in the injectate and hepatic vein samples.

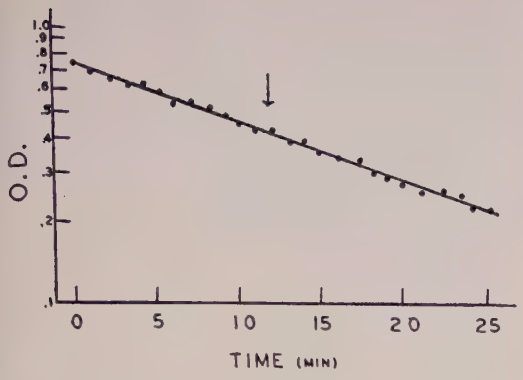

FIG. 2. Typical carbon clearance study. No interference with clearance of carbon particles from the blood occurred following inj. (↓) of haptoglobin hemoglobin complex.

Results. During the first 20 minutes following injection, concentration of the labelled haptoglobin complexes and α_2 globulins did not decrease sharply (Fig. 1a), and the Fe^{59} Hb label disappeared from the plasma exponentially with a mean half time of 110 ± 37 minutes (SD) (Fig. 1b). Studies with HpHb Fe^{59} were limited to an 8 hour period due to the rapid labelling of erythrocytes with Fe^{59} released from the complex. Following injection, plasma concentration of I^{131} HpHb decreased more or less exponentially with a mean half time of 78 ± 26 minutes (SD) reaching levels of 5-20% of initial plasma concentration after 6-8 hours (Fig. 1 b).† Thereafter the rate of fall of plasma I^{131} decreased and the plasma decay curve demonstrated a slower component lasting 1 to 3 days (Fig. 1c). A similar slow component was observed in 3 rabbits injected with I^{131} labelled α_2 globulins (Fig. 1 c). Three to 4 days after injection, the residual plasma radioactivity from both I^{131} HpHb and I^{131}-labelled α_2 globulins rapidly disappeared (Fig. 1c), suggesting development in the rabbit of antibodies against the human proteins. No TCA precipitable radioactivity was recovered in the urine.

Role of liver and reticuloendothelial system in metabolism of haptoglobin hemoglobin complex. Injection of haptoglobin hemoglobin complex into 3 rats during the steady state of carbon clearance had no effect on the latter (Fig. 2). Thus, in the amounts used, there was no evidence that the complex significantly influenced clearance of carbon particles by the reticuloendothelial system. In 2 studies in rabbits in which I^{131} rabbit serum albumin and HpHb Fe^{59} were injected into the portal vein, the ratio of Fe^{59} to I^{131} in the injectate and hepatic vein blood were 1.01 and 1.02 respectively. Thus no evidence for preferential removal of the Fe^{59} labeled complex was obtained in one circulation through the liver. Similar studies with albumin-I^{131} and P^{32} labelled erythrocytes had demonstrated no selective removal of albumin during a single passage through the liver(9).

Discussion. These results indicate that haptoglobin combined with Fe^{59} hemoglobin is removed from the plasma of the rabbit with a half time of about 110 minutes. While the results with I^{131}HpHb are more difficult to interpret because of the presence of small amounts of α_2 globulins, they are sufficiently similar to suggest that the complex is cleared as a whole. The slightly faster plasma disappearance of I^{131}HpHb may reflect some denaturation of the protein during iodination and electrophoresis.

While there may be some reservation in accepting the data obtained with heterologous I^{131} HpHb for these reasons, the close similarity in the fate of both labels suggests that the iodinated protein may also be used to measure endogenous haptoglobin metabolism. Furthermore, recent studies by Murray and associates(10) employing rabbits whose haptoglobin had been raised by injection of turpentine have also yielded comparable values. While the present studies suggest that the complex is removed *in toto*, they fail to characterize its sites of removal or degradation. If the reticuloendothelial system should be shown to play a role, its low affinity for the

† The exact interpretation of the plasma decay curves of I^{131} HpHb would necessitate measurement of the space of distribution of the contaminating I^{131} α_2 globulins. While this is not known, an approximation may be obtained by assuming the same distribution as for albumin, namely an eventual intravascular-extravascular ratio of 40-60. This correction was applied in 4 of the 8 I^{131} HpHb curves, where there were sufficient data. Graphic analyses of the curves resulted in a decrease in mean half time of about 10 minutes.

haptoglobin hemoglobin complex, when small amounts are administered, suggests that a minor component, possibly the bone marrow, may be primarily involved. A final answer to these questions will have to await studies by which the complex can be localized in tissues.

Summary. 1. Haptoglobin hemoglobin complex labelled with Fe^{59} or I^{131} injected into 13 rabbits was removed from the blood with half times of 110 minutes and 78 minutes respectively. 2. The complex did not significantly interfere with phagocytosis of carbon particles by the reticuloendothelial system and was not selectively removed by the liver during a single passage.

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Incorporation of Selenium-75 into Dog Hair. (26048)

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With the proposal that selenium be included among the essential dietary elements (1), interest has been focused on many unanswered questions about the metabolism and fate of selenium in the mammalian organism, especially the chemical nature of selenium compounds in animal tissues. The apparent incorporation of trace amounts of selenium in various tissue proteins has been discussed(2, 3). Continuing these studies, hair which is high in cystine content was examined for selenium incorporation. Hair from Se^{75}-injected dogs was fractionated and assayed for Se^{75}, which was found when cystine-rich protein keratin was isolated as the thiosulfate, S-sulfokerateine. The cystine fraction isolated from the hair by conventional methods contained Se^{75}.

Methods and procedures. The method of Swan(4) was used for the specific and symmetrical fission of disulfide bonds in keratinous tissue. The cystine disulfide bonds in dog hair containing Se^{75} were broken by the reaction with cupric-ammonium sulfite, as illustrated in the equation:

$$RSSR + 2\,Cu^{++} + 2\,SO_3^{--} \rightarrow 2\,RSSO_3^{-} + 2\,Cu^{+}$$

The protein S-sulfokerateine was precipitated from the copper reagent extracts of hair containing Se^{75} by acidification and dilution(4). The protein was then washed repeatedly with water, lyophilized, weighed, and assayed for Se^{75}. A small fraction of the hair that was insoluble in the copper reagent was separated by centrifugation, washed thoroughly with water, lyophilized and counted. The uniformity of the S-sulfokerateine preparations was established by paper electrophoresis analysis. Samples of S-sulfokerateine were dissolved in Veronal buffer (pH 8.6; μ 0.075) and applied to Schleicher and Schuell 2043-A mgl. electrophoresis paper. A constant current of 7 ma was applied for 6 hours. The protein fractions were developed using the standard alcoholic bromphenol blue method. In another experiment, cystine from hair containing Se^{75} was isolated by the method of Gortner and

TABLE I. Selenium Administration.*

Dog	Sex	Wt, kg	Se^{75}, mc	Selenium, mg
1	♂	19.5	.61	.37
2	♂	26.8	.61	.37
3	♀	19.5	1.97	2.50
4	♂	20 4	.84	2.03
5	♂	30.3	1.40	2.14
6	♂	20.4	1.12	.41

* Se^{75} was given subcut. as $H_2Se^{75}O_3$, except that Dog 5 received $Se^{75}Cl_4$.

Hoffman(5), and assayed for Se^{75}. The cystine fraction was recrystallized twice, and qualitative tests indicated that tyrosine was not present. The cystine preparations were chromatographed in both one and two dimensions. Two solvent systems were employed: a *tert*-butyl alcohol-formic acid-water mixture (Solvent I); and a single phase phenol-ammonia-water mixture (Solvent II)(6). Whatman filter paper No. 3 MM was used, and the chromatograms were developed with the ninhydrin reagent of Underwood and Rockland(7).

In a time-distribution study, dogs were injected with trace amounts of Se^{75} and the hair was assayed for Se^{75} at various times after injection. The hair was washed with detergent, rinsed thoroughly with water followed by 95% alcohol and ether. The air-dried hair samples were weighed and assayed for Se^{75} in duplicate. In another experiment, 4 dogs were given single injections of selenium, and after several months comparisons were made of concentration of Se^{75} in hair and in the several tissues. Table I shows amounts of Se^{75}, selenium, and the form of selenium administered to the various dogs.

Results. Radioactive assay of the hair protein, S-sulfokerateine, obtained from 2 dogs at various time intervals after administration of Se^{75} is presented in Table II. About 42% of the original weight of the hair was obtained as the thiosulfate, S-sulfokerateine. About 18% (Dog I) and 16% (Dog II) of the original hair Se^{75} activity were present in the organic thiosulfates. The percent of total hair Se^{75} activity in the insoluble fraction (in copper reagent) for 12 determinations was 10.9 ± 3.1%. Paper electrophoresis studies of S-sulfokerateine showed one component which had a mobility approximately the same as that of dog serum albumin. Results from paper chromatographic studies of the cystine fraction isolated from hair revealed that only one spot was present, with no other detectable amino acids. The fact that specific activity of the cystine fraction showed no significant change between first and second crystallizations indicated high purity of the sample. Se^{75} activity in the cystine fractions of hair containing Se^{75} from 4 dogs is shown in Table III. The hair in each experiment was obtained from different dogs, and was taken at different time intervals after Se^{75} administration. Se^{75} activity ex-

TABLE II. S-sulfokerateine Containing Se^{75} from Dog Hair.

Dog 1					Dog 2				
		S-sulfokerateine					S-sulfokerateine		
Wk after inj.	Specific activity of hair, cpm/g	% of hair Wt	% of hair Se^{75}	Specific activity, cpm/g	Wk after inj.	Specific activity of hair, cpm/g	% of hair Wt	% of hair Se^{75}	Specific activity, cpm/g
3	4800	36.0	20.3	2750	3	2030	30.9	22.6	1070
4	1790	29.5	23.2	1410	7	21940	37.1	10.4	6090
8	12380	41.7	12.9	3890	9	14950	50.8	8.1	2480
9	10420	48.1	10.5	2270	10	7260	46.9	8.8	1370
10	8040	57.6	14.3	2010	11	43760	51.6	16.8	14230
11	21400	55.1	18.8	7300	12	50480	61.8	14.2	11580
12	20600	59.4	19.5	6780	14	21240	37.7	18.9	10670
13	15490	45.4	20.0	6840	15	26810	42.3	13.6	8650
14	12410	35.8	20.3	7030	16	15230	46.8	20.2	6560
15	15330	42.5	23.2	8370	17	8220	34.4	20.9	4990
17	9250	37.4	19.6	4850	18	8500	31.7	18.8	1560
18	9950	31.6	11.9	3770	19	4650	35.1	12.9	3560
19	12670	30.3	19.7	8210					
Avg		42.3 ±8.3	18.0 ±3.5		Avg		42.3 ±7.8	15.5 ±4.2	

TABLE III. Cystine Fraction from Dog Hair Containing Se^{75}.

Exp.	Hair: Wt, g	Hair: Total activity, cpm	Specific activ. cystine fraction: 1st crystallization, cpm/g	Specific activ. cystine fraction: 2nd crystallization, cpm/g	cpm Se^{75} cystine fraction / cpm Se^{75} hair, %
1	200	330,000	2187	2220	1.41
2	100	119,000	1408	1440	2.70
3	50	104,000		2270	2.79
4	108	425,000		6720	7.72

pressed as c.p.m. per g of cystine varied from 1.4 to 6.7 x 10^3 c.p.m./g and 1.4 to 7.7% of the original hair Se^{75} activity was found in the cystine fraction.

Time-distribution studies of Se^{75} in dog hair are shown in Table II, columns 2 and 7, where specific activity of hair (c.p.m./g) is expressed against time. Concentration of Se^{75} in hair gradually reached a maximum around the 12th week, then decreased. Table IV indicates that amounts of Se^{75} in hair, on a dry-weight basis, were as high or higher than in other tissues examined on a wet-weight basis.

Discussion. The long life span, the coherent hard keratinous structure of hair, and absence of apparent metabolic turnover in the hair shaft permitted incorporated selenium to be retained for long periods, as demonstrated in time-distribution studies. Significant amounts of selenium were deposited and retained in hair when compared with other tissues of the animal organism. Keratin, the principal protein of hair, when isolated as the thiosulfate, S-sulfokerateine, contained about 17% of original hair Se^{75} activity. The values 1.4 to 7.7% of the original hair Se^{75} in the cystine fraction represent minimum values, since our experience has been that losses of cystine and Se^{75} occur in hydrolysis, norit treatment, and recrystallization procedures (2). The concept that the cystine fraction of tissues isolated from animals treated with Se^{75} contains an isomorphic mixture of cystine and the selenium analogue, selenocystine, has been discussed elsewhere(3), and presumably applies in the present hair experiments.

TABLE IV. Distribution of Se^{75} in Various Tissues.

Dog	Days after inj.	Hair	Toenails	Kidney	Liver	Spleen
		cpm/g				
3	141	250,000	57,300	28,700	20,200	14,260
4	267	7,000	2,700	2,810	9,560	2,270
5	310	3,560	4,500	1,110	5,320	361
6	316	2,250	1,500	5,300	3,460	2,340

Because of the great reactivity of certain elements with sulfhydryl groups of the hair follicular proteins, the elements have a special affinity for hair(8). These may combine with the sulfhydryl groups of the proteins of the matrix cells as in the case of selenium, copper and arsenic. However, selenium may be converted into either or both selenocystine and selenomethionine in tissues other than hair by some unknown reaction, then incorporated into the protein of hair. The possibility exists that organoselenium from serum proteins may be utilized for hair protein formation, since it has been established that selenium is incorporated in serum proteins(9, 10). Related experiments by Fleischer *et al.*(11) indicate that utilization of C^{14}- and S^{35}-tagged plasma proteins for hair protein formation involves breakdown of plasma proteins to amino acids or small peptides. In view of the affinity of selenium for sulfhydryl compounds and cystine, it has been assumed by workers in the field that selenium enters into chemical combination with keratin(12, 13,14,15). Further elucidation of this concept is presented in this report which shows first, that Se^{75} is incorporated into keratin when this protein is isolated as the thiosulfate, S-sulfokerateine; and second, that Se^{75} is found in the cystine fraction of hair. The above experimental data are presented as additional evidence to that already reported(2, 3) that selenium may exist as selenocystine in tissue proteins.

Summary. Trace amounts of Se^{75} injected subcutaneously into dogs as either $Se^{75}Cl_4$ or $H_2Se^{75}O_3$ were deposited and retained in hair for as long as 316 days. Se^{75} was found

in the cystine-rich protein keratin when the latter was isolated as the thiosulfate, S-sulfokerateine, and in the cystine fraction isolated from hair. The source of selenium which is incorporated into hair protein is discussed.

The technical assistance of Benjamin R. Larke is gratefully acknowledged.

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Effect of Bladder Distension on the Venous System of Man.* (26049)

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Forty years ago Wernöe(1) observed that with certain painful visceral diseases "anemic zones" developed in the skin overlying the affected organ. He attributed the pallor to "ischemia" secondary to vasoconstriction. Stürup(2) described areas of hyperalgesic pallor in the skin of patients with acute appendicitis. Using a photometer Adams-Ray and Nórlen and Adams-Ray(3,4) observed that upon distension of the bladder, pallor developed in the cutaneous ventral segments of T_{11} and T_{12}. They believed the pallor to be due to a spinal reflex with the afferent fibers passing through sacral nerves S_2, S_3 and S_4 and efferent fibers traveling with the sympathetics producing vasoconstriction in the subcapillary venous plexus.

The present study was undertaken to learn the extent of this reflex by studying the effect of bladder distension on an intact isolated venous segment of the forearm of normal and diseased subjects.

Materials and methods. Twelve subjects ranging in age from 27 to 58 years (mean, 36 years) were studied; 5 were male and 7 were female. The cardiovascular system was normal in all but 2 subjects. One subject had luetic heart disease and chronic congestive heart failure and another had transection of the spinal cord at T_6 secondary to metastases from pulmonary carcinoma.

Venous pressure was measured in an isolated intact venous segment and in an adjacent vein open to the systemic venous system of the forearm as previously described(5,6). The pressures were recorded simultaneously and continuously by means of Statham strain gauge pressure transducers and a suitable multichannel direct writing recorder.

A straight French catheter was inserted into the bladder of the subject and was connected by means of rubber tubing, which led out of the subject's room, to a calibrated glass

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bottle containing 500 cc of isotonic saline solution warmed to body temperature. Vesical pressure was also recorded continuously. After insertion of the catheter into the urinary bladder all tubing was shielded from the subject's view so that he was unaware of the flow of fluid into his bladder.

The bladder was emptied and base line levels of the 3 pressures were recorded. Fluid was allowed to flow slowly into the bladder in 50 cc increments. After each 50 cc increment sufficient time was allowed for stabilization of any change in venous pressure. During these experiments care was taken not to disturb the subject. Conversation and activity in the observation room were restricted to a minimum.

Results. 1) *Normal subjects.* In all of the normal subjects the initial instillation of 50 cc of isotonic saline into the empty bladder produced an increase in segmental venous pressure. The magnitude of this rise varied considerably, the range being 20 to 445 mm H_2O with a mean of 120 mm H_2O. The rise in segmental venous pressure was transient, the pressure returning to the base line within 1 to 3 minutes after reaching the peak (Fig. 1). With each additional instillation of 50 cc of fluid, a similar increase in segmental venous pressure occurred; the increase in pressure was usually of essentially the same magnitude as the initial rise (Fig. 1). When the contents of the bladder reached between 300 and 350 cc, most of the patients experienced discomfort and the segmental venous pressure remained elevated until the bladder was emptied. In 4 subjects there was a slight increase in pressure in the systemic veins but the increase never exceeded 25 mm H_2O.

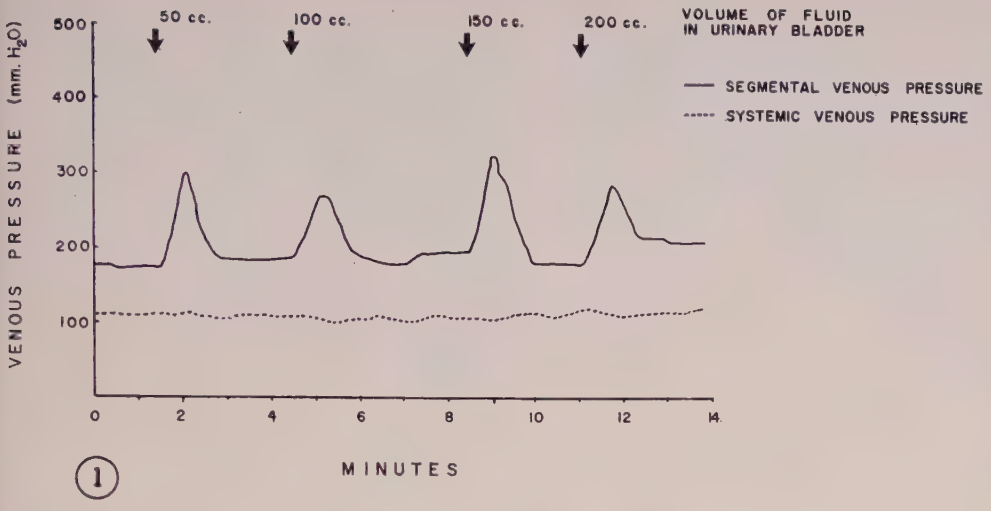
In 3 subjects in whom the nerve supply to the venous segment was interrupted by regional nerve blocking with procaine, there was no response of the isolated venous segment to instillation of the fluid into the urinary bladder (Fig. 2), whereas there was a slight increase in systemic venous pressure in 2 of the subjects.

2) *Patient with congestive heart failure.* The segmental and systemic venous pressures in this patient were elevated initially, basal levels of pressure being 776 mm H_2O and 380 mm H_2O respectively. When 50 cc of fluid was instilled into the bladder, there was a marked rise in segmental venous pressure (Fig. 3). When the pressure reached 1,000 mm H_2O the bladder was drained and the venous tone decreased. Instillation of 50 cc of fluid into the bladder a second time was again followed by a marked increase in segmental venous pressure. Systemic venous pressure increased sharply on both occasions. Coincident with the second increase in venous pressure, severe dyspnea and bubbling rales developed in both lungs so that it was necessary to treat the patient for acute pulmonary edema. Fig. 3 demonstrates the response of the segmental and peripheral veins to bladder distension as well as to various procedures employed in therapy.

3) *Patient with complete transection of spinal cord at the level of* T_6. There was no change in pressure in the isolated venous segment in this patient even when the urinary bladder was filled to capacity (Fig. 4).

Discussion. These observations indicate that slight filling of the urinary bladder can increase venous tone in the forearm veins of man. Since approximately 80% of total blood volume is contained in the venous system, changes in volume of the superficial veins should have a profound effect on the circulation.

The vesico-cutaneous reflex described is probably a spinal reflex mediated through receptors in the wall of the urinary bladder and carried by afferent nervous fibers through sacral nerves S_2, S_3 and S_4. Whether or not there are special higher venomotor centers in the spinal cord and cerebral cortex that control venous tone is not known. The afferent impulse may either pass through such centers or travel more directly to the veins by way of efferent sympathetic fibers from the spinal cord, or both. The reflex nature of the increase in peripheral venomotor tone is evident by the fact that the venomotor response to bladder distension was eliminated by regional nerve block. In addition, the failure of bladder distension to alter segmental venous pressure in the patient with transection of the spinal cord further supports a spinal reflex mechanism.



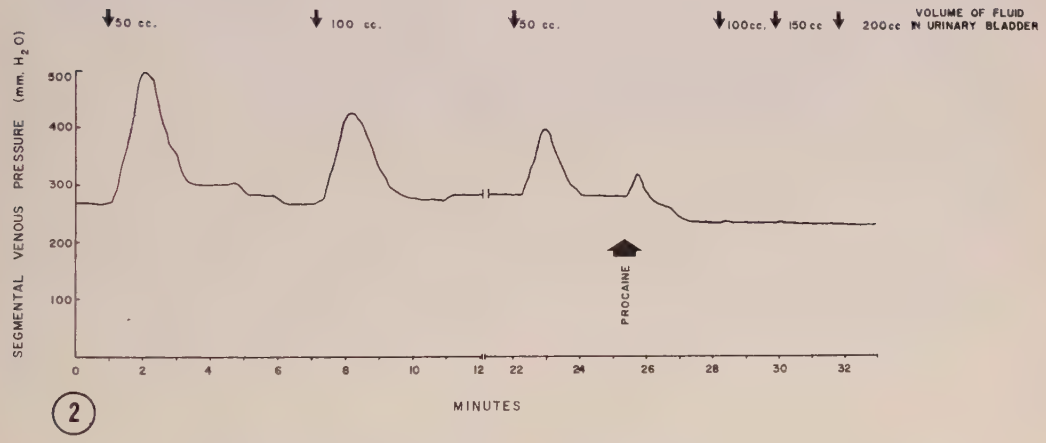


FIG. 1. Effect of instillation of fluid in 50 cc increments into the urinary bladder on segmental and systemic venous pressure.

FIG. 2. Effect of regional procaine block of nervous supply on response of the isolated venous segment to bladder distension. The sharp rise in pressure at time of procaine block was in response to pain and psychic stimulation associated with the performance of the block.

The increase in segmental venous pressure was not due to pain because the venomotor response occurred before the subjects developed discomfort or pain. However, it was intensified by pain, thus indicating again an increase in segmental venomotor tone in response to another type of pain(5,6), namely visceral pain.

It is characteristic of vesical pressure to remain low until the bladder contains a critical volume of fluid, usually about 300 cc in our subjects. However, an increase in segmental venous pressure developed well before such a critical volume of fluid was instilled into the bladder. Even though there was little or no increase in vesical pressure, an increase in segmental venous pressure occurred. Coincident with each instillation of fluid into the urinary bladder there was a slight rise in vesical pressure; adaptation to the new volume of fluid developed quickly, however, and vesical pressure returned to the original level within seconds. It is probable that stimulation of the stretch receptors in the wall of the urinary bladder initiated the venomotor reflex described.

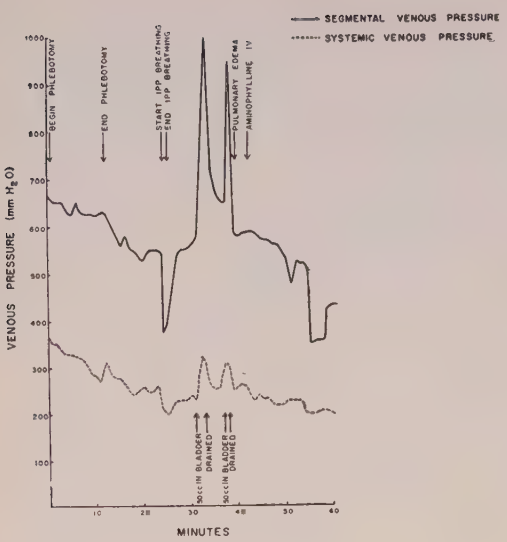


FIG. 3. Effect of bladder distension on venomotor tone in a patient with chronic congestive heart failure. (See text for details.)

Although there was a significant rise in segmental venous pressure with bladder filling in the normal subjects, there was only occasionally a slight rise in systemic venous pressure. The failure to obtain a rise in systemic venous pressure was probably due to the fact that the venous system was "loose" and could accommodate the blood squeezed centrally by constriction of the smaller veins, and/or other portions of the systemic venous system underwent a decrease in tone to compensate for an increase in tone in the superficial veins. However, in the patient with congestive heart failure who already had high venous and sympathetic nervous system tone, there was a considerable increase in peripheral systemic venous tone and pressure. The shifting of blood from the small veins into the pulmonary vessels and veins probably precipitated the pulmonary edema that developed. In this patient the central venous system was already "tight" so that it could not properly accommodate the blood "squeezed" centrally by peripheral venoconstriction. The vesicovenomotor reflex may have also involved the pulmonary veins, arteries and capillaries(7).

After each experiment close interrogation of the subjects indicated that 3 of them felt "something move" in the perineum as the fluid was instilled but felt nothing in the bladder. The remaining subjects felt nothing until the bladder was filled to the point of discomfort and pain.

Summary. Venomotor responses of an intact isolated superficial venous segment of the forearm of man to bladder distension showed that instillation of 50 cc of fluid into the empty bladder resulted in an increase in venous pressure and venomotor tone. The increase in segmental venous pressure could be inhibited by regional procaine block. The response was absent in a patient with complete transection of the spinal cord. Thus, distension of the urinary bladder initiates a spinal venomotor reflex which produces an increase in peripheral venous tone in the super-

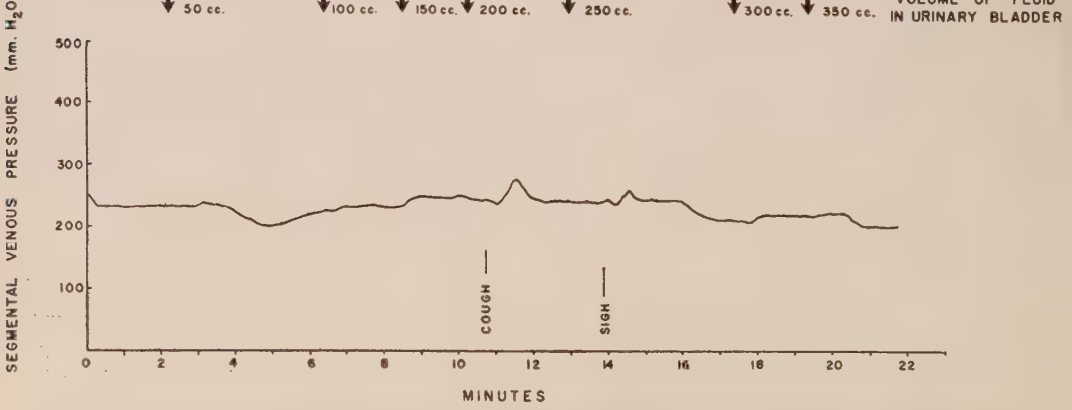


FIG. 4. Failure of bladder distension to influence venomotor tone in a patient with complete transection of spinal cord at level of T_6.

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Changes in Serum Complement Activity in Patients with Myasthenia Gravis.* (26050)

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It has frequently been suggested that the deficit in neuromuscular transmission characteristic of patients with myasthenia gravis might be produced by a curare-like agent which can circulate in the blood. In a search for such an agent, previously described(1), serum samples obtained from a series of individuals afflicted with myasthenia gravis were tested for neuromuscular blocking action. The assay involved measurement of the changes in muscle tension output of an indirectly stimulated frog sartorius muscle-sciatic nerve preparation which was immersed in the diluted serum.

Serum samples obtained from a few myasthenic patients caused a reduction in muscle tension output, and this result appeared to parallel the cytolytic destruction of fibers lying on the surface of the sartorius muscle used in the assay(1). From these and more recent observations(2), it is clear that serum exhibiting the above type of cytolytic activity occurs in 44% of patients afflicted with myasthenia gravis and in 22% of normal controls. The relative strengths of the active serums found in each group are unknown.

The results described above led us to think about the nature of the cytolytic system involved and the possible connection between the activity of this system and etiologic factors which operate in myasthenia gravis. As to the nature of the cytolytic system, we speculated that serum complement (C′) might be concerned because, as is well known, C′ plays an essential part in immune hemolysis. For this and other reasons, it seemed worthwhile to carry out determinations of C′ activity on serum obtained from myasthenic patients. Encouragement was provided by the early results which showed that in many myasthenic patients, serum C′ activity was far outside the normal range.

In this paper we have reported results of serum C′ analyses performed on samples collected from a large series of patients with myasthenia gravis. Many of these individuals were studied for long periods, with particular attention to changes in serum C′ activity and clinical condition. A brief survey

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We owe much to Mrs. L. V. Berk, Mr. J. T. Alexander and Mrs. Elaine Shapiro for their faithful, careful efforts in the assay work and the serum collection program.

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of this work has been given previously (Nastuk *et al.*(3).

Methods. a) *Patients and controls.* The patients with myasthenia gravis who cooperated in this study showed considerable variation in severity of disease symptoms and in number and distribution of affected muscles. All patients were aided by adminstration of anticholinesterase drugs (usually pyridostigmine). The series of myasthenic patients includes cases in which a thymoma was present, in which therapy and treatment in addition to anticholinesterase drug administration were employed, and in which other clinical conditions of a possible complicating nature arose. Our rules for dealing with these situations are stated in the section under results concerned with clinical correlation.

Serum samples representing the normal control group were obtained from 4 females and 9 males all in good health and ranging in age from 20 to 50 years. From 2 of the females, samples were secured 1 to 4 days prior to beginning of menstruation and 1 to 2 days following cessation of menstruation.

b) *Blood sampling and storage.* At the start of this investigation, we specified withdrawal of medication from each myasthenic patient for a period of 12 hr prior to obtaining a blood sample. Adherence to this rule was difficult with severely afflicted patients who experienced distress if drug administration was stopped for more than 4 hr. Being forced therefore to draw blood samples at relatively short intervals following drug administration, we performed the following experiments to assure ourselves that the patient's serum C′ activity, as we determined it, was not influenced by his anticholinesterase intake.

Addition of pyridostigmine (10 mg/l) to serums obtained from a normal individual and a patient with myasthenia gravis had no effect on C′ activity assayed as described herein. This concentration of pyridostigmine is greater than that likely to be present in the serum of myasthenic patients receiving the drug.

In 5 myasthenic patients, C′ activities were determined on serum samples obtained before and during a 4 hour period following administration of pyridostigmine. No significant change in serum C′ activity was found.

A possibility not excluded by the preceding experiments is that prolonged administration of anticholinesterase medication causes slowly developed changes in serum C′ activity. We have no evidence bearing on the validity of this speculation, but even if correct, it does not provide a sufficient basis to explain our data. This point will be more fully treated in the discussion.

Details of blood sampling and storage procedure are as follows: without exercise or other special maneuvers, 10 to 20 ml of blood was drawn from the antecubital vein and placed in Pyrex centrifuge tubes which were covered with a sheet of Parafilm. After about 30 min., the clotted sample was centrifuged at 3000 rpm for 30 min., the serum was drawn off and placed in another Pyrex centrifuge tube and was tightly sealed with a sheet of Parafilm or, in later work, with a rubber stopper.

For 90% of the samples collected, the above procedures were completed within 3 hr whereupon, until removed for analysis, these serum samples were placed in a freezer and maintained at –35°C or lower. The remaining 10% of the samples, most of which were collected early in the study, received the same treatment except that they were stored one to 7 days at 0°C before final transfer to the freezer maintained at –35°C. Using serum from a normal individual, we were unable to detect a significant loss of C′ activity in an aliquot stored 7 days at 4°C. Therefore, we accept as valid C′ activities determined on those samples which were subjected to interim storage at 0°C.

c) *Analysis for serum complement activity* (4). 1. All glassware was carefully cleaned using a detergent solution (Alconox) followed by sulfuric acid-sodium dichromate solution, then was thoroughly rinsed and dried in an oven.

2—Stock sheep erythrocyte suspension. To 125 ml of a modified Alsever's solution (containing 2.45 g glucose, 2.2 g sodium citrate and 0.8 g citric acid per 125 ml solution) was added 375 ml of freshly drawn sheep's blood. After transport from the local source,

the treated blood was filtered through gauze into a flask which contained 2 x 10^6 units of Penicillin G potassium, and 25 ml of disodium versenate solution. The latter solution was prepared by making up 0.15 M disodium versenate (disodium salt of ethylenediaminetetraacetic acid) and adding sufficient NaOH (1M) to raise the pH to 7.5. The thus preserved sheep erythrocyte suspension was stored in a stoppered flask at 4°C for 1 week before use, and was discarded after 5 weeks storage.

3. Veronal buffer. A stock solution was prepared by dissolving 5.75 g 5, 5-diethyl barbituric acid in 500 ml hot water. This was added to a solution containing 3.75 g sodium 5, 5-diethyl barbiturate and 85 g. NaCl and the mixture was diluted with water to 2 liters. For C′ determination, 50 ml of the above stock was mixed with 0.25 ml of 0.15 M $CaCl_2$, and 0.25 ml of 0.5 M $MgCl_2$. The mixture was diluted with water to 250 ml. The resultant buffer solution, called "veronal buffer" in the following text, was used for all subsequent washings and dilutions. It contains optimal concentrations of calcium and magnesium(5).

4. Standard red blood cell suspension. Approx. 20 ml of the thoroughly mixed stock sheep erythrocyte suspension was placed in a 40 ml centrifuge tube which was capped and spun at 3000 rpm for 5 min. The supernate was withdrawn and sufficient chilled (0°C) veronal buffer added to fill the tube which was then recapped, mixed and centrifuged as before. The washing procedure was repeated 5 times or more until the supernate was free of hemoglobin and cell fragments. A 7 ml aliquot of the lightly packed cell suspension was taken and mixed with 50 ml of chilled veronal buffer after which the mixture was filtered through a cotton plug and again chilled to 0°C. After thorough mixing of this suspension, a 1 ml sample was taken and diluted to 25 ml with water, which resulted in lysis of the erythrocytes.

Optical density of the lysate was determined at 541 mμ using a Beckmann DU spectrophotometer with square Pyrex cuvettes having a 10 mm light path, water serving as the blank. The O. D. obtained (always greater than 0.816) was used to calculate the amount of veronal buffer required to adjust the rbc suspension to the desired value of 2 x 10^9 cells/ml. Following this procedure, a lysate produced from an rbc suspension containing 2 x 10^9 cells/ml will have an O.D. of 0.816(4).

5. Sensitization of standard rbc suspension. The rabbit anti-sheep erythrocyte serum was appropriately diluted (see below) with veronal buffer (0°C). To the standard rbc suspension (at 0°C) an equal volume of the diluted anti-serum was added while mixing. The rbc suspension was then incubated for 30 min with shaking in a water bath at 37°C. The suspension now contained sensitized erythrocytes in a concentration of 1 x 10^9 cells/ml. One ml of this mixture was diluted with water to 25 ml and optical density of the lysate was determined as before. Slight deviations from 0.408, the expected value, were taken into account in the final calculations.

The dilution factor for any particular lot of rabbit anti-serum was set such that the suspension of sensitized sheep erythrocytes, when prepared as described above, contained twice the concentration of antibody required for maximal sensitization of the cells. Determinations of extent of sensitization were carried out with normal serum whose C′ activity was close to the value representing the mean for a group of normal individuals.

6. Analysis for serum C′ activity-general procedure. All solutions and glassware were kept at 0°C unless otherwise indicated. To a 2 ml aliquot of each serum sample appropriately diluted with veronal buffer (see Sect. 7 and 8 below), 10 ml of standard sensitized erythrocyte suspension was added and mixed. Two ml aliquots of this mixture were added to each of 3 chilled test tubes which were then placed in a water bath at 37°C and shaken continuously. After 60 min incubation, one test tube was removed, quickly chilled to 0°C and centrifuged at 3000 rpm for 4 min. Without delay, most of the supernate was decanted into a clean tube, taking care to avoid transfer of erythrocytes. One ml of this decanted solution was pipetted into a clean tube to which 3 ml of water had been added. The

mixture was thoroughly stirred. After 90 and 120 min elapsed incubation time, the second and third tubes were removed from the water bath and similarly treated. Optical density of each final solution was determined as discussed earlier in connection with the standard rbc lysates.

For each serum dilution thus analyzed, optical density determined at various durations of incubation was plotted *vs.* time and from this plot an estimate was made of the O.D. which would be obtained at completion of the reaction. In practice, the slope of these plots was small and the estimated O.D. was usually very close to, if not equal to, the O.D. obtained for the sample incubated for 120 min. We therefore soon adopted the practice of incubating for 120 min. only, and considered that readings taken at this time represented the endpoint of the reaction. Final O.D. readings obtained by either method were corrected by subtracting the O.D. obtained in appropriate blank determinations in which the diluted serum was omitted.

7. Calibration of analysis for serum C′ activity. In this work, serum C′ activity was determined on the basis of degree of lysis produced in a standard suspension of sensitized sheep erythrocytes. Many analyses were carried out, over a long period. Therefore to maintain the internal consistency of our data, we required a calibration of each freshly prepared standard rbc suspension. The primary C′ standard used for calibration purposes was a large stock of serum obtained from a healthy human donor. Five ml aliquots of this serum were kept in rubber stoppered Pyrex tubes held at −35°C in the freezer containing all other serum samples.

Fig. 1 shows a typical calibration curve in which degree of lysis is plotted against a range of C′ concentrations (serial dilutions of the standard serum). Each time a group of serum samples was analyzed, a similar plot was prepared. The plots show that the 10-fold diluted serum standard produced, on different occasions, degrees of lysis ranging from 0.5 to 0.6, indicating that the various factors in the analysis were under reasonable control.

8. Determination of the relative C′ activity of individual serum samples. Each serum sample was diluted 10-fold with veronal buffer and the degree of lysis produced by an aliquot of this diluted serum was determined as outlined in Sect. 6. If the degree of lysis obtained did not fall on the central portion of the calibration curve obtained with the serum standard, the determination was repeated using an appropriately adjusted dilution of the serum sample. In practice, the dilutions required ranged from 0 to 30-fold.

Mean normal value of serum C′ activity was obtained by averaging C′ activities of 29 control samples obtained from 13 normal individuals (see results). Our standard normal serum which was used to construct the calibration curves had a C′ activity equal to this mean normal value, therefore the curve could be used to calculate directly the C′ activity of a particular serum, relative to mean normal C′ activity. We were fortunate in our choice of the normal serum standard; however any normal serum could have been used after determination of the appropriate scaling factor.

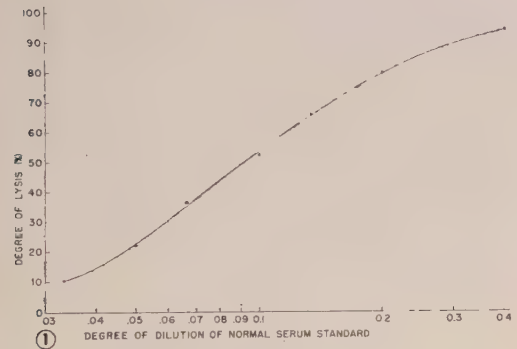
A simple method of dealing with the problems of standardization and determination of relative C′ activity might be to pool serum samples obtained from a large group of normal individuals and to use this pool as a source of standard serum C′ having unit activity.

Results. 1. Control series. Serum C′ activity was determined for 32 samples obtained from 13 normal persons (9 males, 4 females) whose ages ranged from 20 to 50 yrs. Average C′ activities for males and females were not significantly different. Tests on 2 of the females showed that menstruation does not affect serum C′ activity. For all 32 serum samples, C′ activities showed a standard deviation of the mean of $\pm$ 2%, and a *maximum* deviation from the mean of $\pm$ 19%.

2. Patients with myasthenia gravis. Serum C′ activities were determined for 68 patients with myasthenia gravis. In 34 cases, 3 to 14 serial determinations were carried out over a time span ranging from 2 to 44 months. In 12 cases, 2 C′ determinations were made over a time span ranging from 1 week to 9 months. In the remaining 22 cases a single C′ determination was made.

Serum C′ activities determined for these

patients were distributed over a wide range. Frequently values were obtained which were below or above the *extremes* of the range of our control series (lower limit = 0.8, upper limit = 1.2 mean C′ activity = 1.0). Forty of the myasthenic patients gave one or more serum samples whose C′ activity was below the lower limit of the control range, and values as low as 0.05 were found. Thirty-one patients gave one or more serum samples which showed C′ activity lying above the upper limit of the controls, the maximum value being 2.57. Evidence for wide variation in serum C′ activity was obtained in 17 patients who at different times showed values below and above the limit of the control range. Fifty-four of the 68 patients at some time showed a C′ activity lying beyond the limits of the control range (Fig. 2).



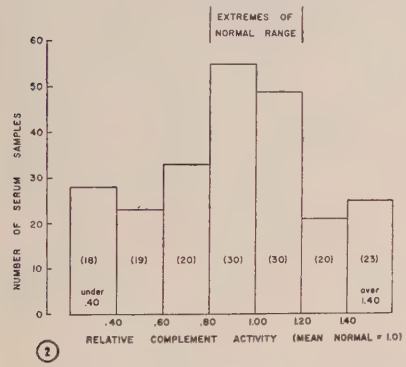


FIG. 1. Typical calibration curve showing relation between degree of lysis of a sensitized sheep erythrocyte suspension and complement activity expressed in terms of degree of dilution of normal serum standard.

FIG. 2. Showing distribution of C′ activities in serum samples obtained from 68 patients with myasthenia gravis. Numerals in parenthesis give No. of patients contributing serum samples whose C′ activities fell in the range indicated.

Clinical correlation. We have surveyed the case histories of the 68 myasthenic patients with special attention to the 3-6 month periods preceding and following the dates on which blood samples were taken. In particular we have tried to determine whether the patient's disease symptoms were increasing in severity or distribution (indicative of an exacerbation), diminishing in severity or distribution (remission) or constant (status quo). The criteria used in setting these 3 categories were: a—substantial changes in anticholinesterase drug intake; b—changes in the patient's work performance and living habits; c—changes in distribution of affected muscles or in severity of disease symptoms in these parts.

Of the entire series of cases, 22 were not included in the assessment of clinical correlation for the following reasons: a—insufficient number of blood samples or insufficient clinical data to establish the disease trend (15 cases); b—X-irradiation to thymus during sampling period (2 cases); c—thymectomy during sampling period (2 cases); d—presence of other disease (3 cases). In the remaining 46 uncomplicated cases, evidence of disease exacerbation was seen in 15. Eleven showed serum C′ activities below the normal range, one fell in the normal range, and 3 were above the normal range.

Evidence of disease remission was seen in 21 of the 46 uncomplicated patients accompanied in 11 of these cases by a rise in C′ activity which reached the supernormal range, 6 showed a rise in C′ activity which reached the normal range, 4 showed no change or a drop in C′ activity.

Fourteen cases were rated as status quo, 6 of these showed C′ activities which were within the normal range and showed little variation. The 8 remaining cases included 3 pregnant women each of whom gave birth to an infant exhibiting neonatal myasthenia gravis. One of these women showed, during the last trimester, a rise in serum C′ activity to supernormal levels. Her symptoms remained essentially unchanged and there was a slight rise in anticholinesterase medication during the course of the pregnancy, a change considered to be not significant. The second

woman showed during pregnancy a serum C′ activity so low that it could not be accurately determined. The third mother showed variable serum C′ activity with all values lying in the subnormal range.

The remaining 5 cases of the status quo group include the following: one patient whose C′ activity was fairly constant but subnormal, one patient whose C′ activity varied between the subnormal and normal range, one patient whose C′ activity showed a marked drop into the low subnormal range, one patient whose C′ activity rose from the high normal to the supernormal range, and one patient in whom the C′ activity remained fairly steady but in the supernormal range.

Discussion. The results reported here show that in patients with myasthenia gravis, serum complement (C′) activity fluctuates over an abnormally wide range. The clinical data indicate that these changes in serum C′ activity are related with exacerbation and remission of the disease.

Our findings direct attention to the possibility that an immune mechanism may play an etiological role in myasthenia gravis. Such an etiological basis would be greatly strengthened if it could be shown that the variations in serum C′ activity reflect the participation of C′ in a mechanism capable of producing the defects seen in myasthenia gravis.

An alteration in serum C′ activity may be produced in a variety of ways such as: (a) change in rate of synthesis, breakdown and excretion of C′, (b) shift in equilibrium between C′ stores and circulating C′, (c) binding of C′ by tissues, (d) disproportionation of C′ components, (e) production of C′ inhibitors. Clearly one might take the position that the changes in serum C′ activity in patients with myasthenia gravis are simply incidental accompaniments of the disease. In such a case, serum C′ changes would have no critical etiological significance but might nonetheless have great practical value in indicating the periods during which the disease process is active. It must be kept in mind that logically speaking, one *need not* consider the etiological process to be active merely because a patient shows a neuromuscular transmission deficit and a positive response to anticholinesterase drugs. On the contrary, there seems to be some reasonable ground for the argument that in many myasthenic patients, the neuromuscular transmission deficit long outlasts the period of activity of the defect-producing agents.

This view is not new. Keynes(6), reporting the effects of thymectomy in a group of myasthenic patients, states that the least postoperative improvement was obtained in those in whom the disease symptoms had been present for the longest periods. Keynes speculated that in longstanding chronic cases there may be present appreciable irreversible damage which sets a limit on the extent of the patient's postoperative remission. In further support of his idea, we may add that our study includes certain myasthenic patients who for long periods of time showed both an unchanging clinical status and a serum C′ activity always within the normal range. Tentatively, we have assumed that the disease process was inactive in these individuals.

One may next take up the point that the changes in C′ activity in myasthenic patients are caused by administration of anticholinesterase drugs. Evidence presented in the methods section indicates that pyridostigmine medication has no short-term influence on serum C′ activity. The drug may also have no long-term influence since certain of our patients showed a rise in serum C′ activity during its prolonged administration. Even more convincing is the fact that we have found subnormal C′ activity in serum of myasthenic patients who had not before received anticholinesterase medication. Our present evidence concerning this point is meager and we hope to extend it.

During early phases of this work we were attracted to the idea that the changes in serum C′ might result from its participation in an auto-immune reaction. The mechanism we visualized was the following. The myasthenic patient is one who has developed an auto-immunity against one of the components (M) of his skeletal muscle fibers. M, an entity found in the plasma membrane or intracellular elements was assumed to have combined with a foreign antigen S and the

combination MS, being antigenic, led to formation of auto-antibodies (A) against it. On reaching the muscle fiber, A would be bound forming MSA, a group which in turn would finally bind C′. Serum C′ would thus be consumed and in its presence there could be produced either a cytolytic destruction of the cell membrane or a subcytolytic alteration in its configuration. From the latter one might obtain a loss in the acetylcholine sensitivity of the postjunctional membrane, inhibition of muscle cell membrane conduction, failure of excitation-contraction coupling etc. Since the characteristic structures localized at the neuromuscular junction have a much increased membrane surface area, the above reaction might for this reason alone be intensified at this restricted site. We further assumed that depending on duration and severity of the above reaction, muscle fibers might be either reversibly or irreversibly damaged and that the terminal arbor of the motor nerve might be indirectly or directly involved.

This scheme has received strong support in explaining the causation of other disease conditions. For example, Roitt and Doniach(7) have presented an excellent summary of the evidence for the presence of auto-antibodies in thyroid disease. Their article provides a good source of the types of experimental attack which we believe must be made in the study of myasthenia gravis. Recent stimulating reviews concerning the participation of C′ in immune reactions are those of Lepow (8), and Osler *et al.* (9).

As is well known(8), serum C′ activity can change in many clinical conditions other than myasthenia gravis but this is of course no argument against its participation as outlined above in our scheme. What one needs is direct evidence concerning the presence and identity of the other components of our postulated system. Detection of the auto-antibody seemed the most critical, and our first attempt to do this was made possible by the kind cooperation of Dr. Seymour P. Halbert, Dept. of Ophthalmology, College of Physicians and Surgeons, Columbia University. Dr. Halbert used the Ouchterlony plate technic to test the reaction between serums obtained from our group of myasthenic patients and several possible sources of antigens including human skeletal muscle. The serums of certain myasthenic patients reacted faintly with the extract of human skeletal muscle but unfortunately the same result was also obtained with serums obtained from a few normal controls. This discouraging failure to obtain a positive result against extract of skeletal muscle might be explained on several grounds other than lack of antibody in the serum. We have since restudied the matter and are now attempting to demonstrate circulating auto-antibodies using the sensitive tanned hemagglutination technic. Very strong encouragement is provided by the evidence in the accompanying paper.

Our other line of evidence that changes in serum C′ activity may be the result of its uptake by an antigen-antibody complex is derived from the unpublished work of Plescia and his colleagues, who determined the activities of the individual C′ components in serums from myasthenic patients and found that C'_1 and C'_3 were normal, and C'_2, C'_4 were greatly reduced. This result is like that obtained in immune C′ fixation(8) but such interpretation may be complicated by presence of inhibitors of the C′ system which Plescia is able to detect.

Finally, in considering the hypothesis that myasthenia gravis is a disease involving a destructive auto-immune mechanism, one may note two additional points. Pathological changes in the skeletal muscle of myasthenic patients are recognized as important features of the disease(10). There is also convincing evidence that the thymus gland is capable of manufacturing antibodies(11), which opens the possibility that the connection between thymoma, thymic hyperplasia and myasthenia gravis is *via* an immune mechanism.

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Immunofluorescence Demonstration of a Muscle Binding, Complement-Fixing Serum Globulin Fraction in Myasthenia Gravis.* (26051)

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Serum from some patients with myasthenia gravis has been shown to have a heat-labile cytolytic effect on sartorius muscle of the frog (1). Serum complement levels in many myasthenic patients in various stages of the disease often vary greatly beyond both the upper and lower limits of normal(2,3). These findings led to the speculation that some complement fixing immunologic system might be implicated in the pathogenesis of myasthenia gravis. In a test of this hypothesis, the immunofluorescence technic of Coons and Kaplan(4) was employed, using serum and skeletal muscle from patients with the disease.

We have demonstrated that the globulin fraction, prepared by 20% sodium sulfate precipitation of serums pooled from 10 myasthenic patients in the early progressive phase of their illness, as distinct from the normal serum globulin fraction prepared in like manner, has the following characteristics: 1. Ability to localize regularly and consistently in alternate striations of human skeletal muscle, both normal and myasthenic, as well as in skeletal muscle of one other mammalian species. This property was made evident by direct tagging of both myasthenic and normal globulins with fluorescein isothiocyanate. 2. Ability, when untagged, to block adherence of fluorescein tagged myasthenic globulin to skeletal muscle. 3. Ability, when once localized in skeletal muscle, to bind whole guinea pig complement. This fixation, *in vitro,* of heterologous complement was demonstrated by use of fluorescein conjugated rabbit antibodies to guinea pig complement. We have also shown that some individual whole myasthenic serums, including some of those utilized in the preparation of the myasthenic globulin pool, as distinct from normal human serums, are (1) capable of blocking adherence of fluorescein tagged myasthenic globulin to skeletal muscle striations, and (2) seemingly capable of fixing guinea pig complement in much the same way as does the myasthenic globulin fraction.

Materials and methods. Source of serums, and preparation of globulin pools. Serums from patients with myasthenia gravis were obtained on the wards and in the outpatient departments of Mount Sinai Hospital, and Columbia-Presbyterian Medical Center, New York. Diagnosis in each case had been established by history, physical findings, symptoms, and positive response to one or more anticholinesterase drugs. Control serums were taken from 12 medical house officers with an age and sex distribution comparable to that of the myasthenic patients. All were essentially healthy and denied histories of major system disease.

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All blood samples were collected with sterile precautions and the serums stored without preservative at 4°C, from 7 days to 6 months before use. Our experience has been that these storage conditions favor preservation of serum globulins suitable for tagging with fluorescein isothiocyanate. When many of these serums were drawn, we did not anticipate their use in a study involving serum complement activity; therefore no attempt was made to store aliquots in the deep freeze to preserve complement titers.

Ten serums were selected from among 105 collected from 96 myasthenic patients. The following criteria were used for selection of serum for this pool: (1) Patients shall have had clinical manifestations of myasthenia gravis for less than 3 years, preferably for less than one year, (2) generalized disease involving bulbar and peripheral musculature shall be manifest, (3) the disease should be progressive or acutely exacerbating at time of blood collection, (4) patients shall have some evidence of thymic pathology by radiography, or as assessed by observation at time of thymectomy. The patients shall not have undergone thymectomy prior to sampling; information regarding thymic pathology seen at surgery would, therefore, be used retrospectively. Seven serums came from patients with a history of clinical disease of no more than one year; the remaining serums from patients with less than a 3 year history of clinically apparent myasthenia gravis. Accordingly, serums from 10 patients were pooled in the following proportions to make a serum pool of 25 cc.

Patient	Age	Clinical course at time of blood collection	Amt of serum, cc
1. ♀	30	3 mo history of oculobulbar and peripheral weakness, positive response to anticholinesterase medication. Thymectomy, 10 days post sampling revealed hyperplastic thymus.	7
2. ♀	53	8 mo history of oculobulbar and peripheral weakness. Positive response to anticholinesterase medication. Thymectomy, one day following sampling, revealed benign thymoma.	2
3. ♂	14	3 yr history of progressive peripheral and oculobulbar weakness. Positive response to anticholinesterase medication. Thymectomy next day revealed thymic hyperplasia.	2
4. ♀	15	1½ yr history of progressive generalized disease. Positive response to anticholinesterase medication. Thymectomy, 5 days post sampling, revealed hyperplastic thymus.	2
5. ♂	26	10 mo history of generalized and progressive weakness. Positive response to anticholinesterase medication. Thymectomy 5 days post sampling revealed benign thymoma.	2
6. ♂	53	2 yr history of generalized disease. Positive response to anticholinesterase medication. One yr prior to sampling, patient underwent exploratory thoracotomy, at another hospital; inoperable thymoma found.	2
7. ♀	32	3 yr history of oculobulbar and generalized disease. Positive response to anticholinesterase medication. Thymectomy, 2 wk post sampling, revealed thymic hyperplasia.	2
8. ♂	48	One yr history of generalized & oculobulbar symptoms. Positive response to anticholinesterase medication. Subsequent thymectomy revealed malignant thymoma.	2
9. ♀	55	One yr history of generalized & oculobulbar symptoms. Positive response to anticholinesterase medication. Subsequent thymectomy revealed thymoma.	2
10. ♀	48	Approximately 6-8 mo history of generalized progressive disease. Evidences of enlarged thymic shadow by x-ray.	2

Aliquots from 10 myasthenic serums were combined as given above to make a 25 cc serum pool; a similar pool was prepared from 12 serums from healthy individuals. Each pool was absorbed with 6 ml of packed sterile human erythrocytes, AB positive, washed 3 times with 0.85% NaCl.

Preparation of globulins from myasthenic and normal serum pools. The serum pools were diluted with equal volumes of sterile 0.85% NaCl and to each 10 ml of serum saline mixture, 2.04 g of anhydrous sodium sul-

fate were added. After standing at room temperature for 2 hours or more, the suspensions were centrifuged and precipitates dissolved in volumes of distilled water equal to two-thirds of initial volumes of diluted serum pools. The globulins were reprecipitated by adding 1.93 g of sodium sulfate for each 10 ml of distilled water added. The suspensions were again centrifuged and a third precipitation carried out in the same manner. Final precipitates were dissolved in minimum amounts of distilled water, dialyzed against running cold tap water until first appearance of reprecipitation, then dialysis was continued against 0.9% NaCl with mechanical stirring overnight at 4°-6°C. The resulting globulin solutions were adjusted to a concentration of 20 mg/ml with normal saline, passed through Seitz Filters, and stored in the refrigerator at 4°-6°C without preservative. Paper electrophoresis of myasthenic and normal serum globulin fractions thus prepared revealed albumen free preparations consisting of alpha, beta, and gamma globulins. Curves for myasthenic and normal pools were completely superimposable. Immunoelectrophoresis of both globulin preparations against horse anti-human whole serum revealed essentially identical results for myasthenic and normal globulin pools.

Portions of both myasthenic and normal globulins were conjugated with fluorescein isothiocyanate‡ according to the methods of Riggs(5) and Marshall *et al.*(6). Fluorescein conjugated globulins were dialyzed with sterile precautions against 0.01 molar phosphate buffered saline (pH 7.2) at 4°-6°C for 6 days to eliminate free fluorescein isothiocyanate. Conjugates were subsequently absorbed with rat and mouse liver powders to remove non-specific fluorescence.

Guinea pig complement. Hyland§ Dried Complement (guinea pig) prepared from healthy male or non-pregnant female guinea pigs was used (Lot #370M26) throughout these experiments. The equivalent of 7 cc of dried guinea pig complement was reconstituted with 5 cc of diluent supplied by Hyland Laboratories. All samples so prepared were assayed by the Serology Laboratories of Presbyterain Hospital, New York, and found to have hemolytic complement activities of 1:42 by dilution. Dilutions used for our studies ranged from 1:15-1:30. A calcium-magnesium saline solution (instructions for preparation accompany packaged dried complement) was used for all complement dilutions.

Fluorescein conjugated rabbit anti-guinea pig complement. This conjugated anti-serum was prepared according to technics described elsewhere(7). Antigen used to prepare this anti-serum in rabbits was sensitized sheep red cell stromata that had been exposed to guinea pig complement. The resulting rabbit anti-serum globulin was absorbed with sheep red cell stromata, and sensitized sheep red cell stromata, free of fixed guinea pig complement, and the resulting anti-guinea pig complement was tagged with fluorescein isothiocyanate.

Collection and preparation of tissues, staining with fluorescent globulin conjugates: The following tissues were utilized in this study:

Human skeletal muscle biopsies.

A. Intercostal muscle biopsy taken at thymectomy from patient #1, a myasthenic who contributed to the serum pool.

B. Forearm muscle biopsy from a 71-year-old female myasthenic.

C. Rectus Abdominis muscle biopsy from a 43-year-old myasthenic undergoing hysterectomy.

D. Forearm muscle biopsy from a 33-year-old female patient with myotonic dystrophy.

E. Pectoralis muscle biopsy from a six-year-old male undergoing open heart surgery for a congenital cardiac defect.

F. Rectus abdominis muscle biopsy from a 43-year-old male undergoing subtotal gastrectomy for peptic ulcer.

Rat skeletal muscle

G. Abdominal skeletal muscle biopsy from a healthy male Sprague-Dawley rat.

Human cardiac muscle

H. Auricular appendage containing myocardium, from patient with rheumatic valvular disease who had undergone mitral commissurotomy.

Human myometrium (smooth muscle)

‡ Sylvana Chemical Co., Orange, N. J.

§ Hyland Labs., Los Angeles, Calif.

I. and J. Two biopsies taken from uteri at hysterectomies from 2 myasthenic women. One of these 2 patients also provided skeletal muscle biopsy C. described above.

Thymic tissue: from 2 myasthenic patients.

K. Thymic hyperplasia from patient #1, who had contributed serum to pool and intercostal muscle biopsy A., described above.

L. Thymoma tissue from patient #9 of myasthenic serum pool.

All tissues were taken at time of surgical removal and quick frozen in sealed Pyrex test tubes surrounded by a slush of dry ice and butanol, then stored in a dry ice freezer at −65°C until use. All tissues were sectioned on an A-O Rotary Microtome at a setting of 1-2 μ in the longitudinal plane in a Harris Cryostat at −20°C. Sections were mounted on 1 mm thick slides as required for dark field observation with an ultraviolet source. Before 'staining' with fluorescein conjugated globulins, sections were fixed in 95% Ethanol for 30 seconds. Alcohol was washed from the slides with 0.01 M phosphate buffered saline, pH 7.2. Slides were then rinsed in buffered saline for 10 minutes, then wiped as dry as possible without removing fluid overlying sections. Slides were then transferred to moist chambers and submitted to the respective control and test staining procedures to be described. Following completion of staining schedules, sections were again rinsed in buffered saline for 10 minutes, mounted in buffered glycerin (pH 7.2), and covered with No. 1 cover slips. All sections were examined by means of an ultraviolet light source using a 200 Watt high pressure mercury vapor arc lamp of 2500 stuble mounted in a Reichert Fluorex Unit. Five filters were used for visualization, 2 red excluding, 1 ultraviolet transmitting, 1 BG 12, and 1 ultraviolet excluding filter over the ocular.

Photography. A Leica Camera Body was used with a Reichert KAM attachment to the microscope. All photographs were taken on AGFA ISOPAN RECORD film, at ASA 4000 under 10 x 43 magnification with an average exposure of 15 minutes.

Results. Sections of skeletal muscle from all human biopsies, normal, myasthenic and dystrophic were treated with the fluorescein conjugated myasthenic globulin fraction, undiluted, or diluted 1:2.5, 1:4, and 1:5, two drops per section, for 30 minutes, and examined following a 10 minute rinse with 0.01 M phosphate buffered saline, pH 7.2. In every section, brilliant apple green fluorescence was observed in alternate striations consistently throughout every longitudinally sectioned fiber (Fig. 1). A more or less punctate fluorescence was seen in fibers cut in transverse plane. In some sections, especially where the fluorescein tagged myasthenic globulin was applied undiluted, there was a variable amount of fluorescence in the sarcolemma. There was no fluorescence of this color value or intensity in the connective tissue stroma or blood vessels included in these sections. Sections stained with fluorescein conjugated normal human globulin fraction *did not* fluoresce in any locus (Fig. 2). Rat skeletal muscle, when treated similarly with fluorescein conjugated myasthenic globulin, evidenced focal localization of fluorescent material in alternate striations. Normal human fluorescein tagged globulin produced no fluorescence in rat muscle. Sections of auricular appendage myocardium failed to evidence fluorescence when treated with either myasthenic or normal fluorescein conjugated globulin fractions, and sections of myometrium were also negative. Sections of thymic tissue from 2 myasthenic patients likewise were not stained by fluorescein conjugated myasthenic and normal globulin fractions.

Several sections of skeletal muscle from each biopsy were treated with myasthenic globulin unconjugated and undiluted, for 15 minutes prior to treatment with fluorescein tagged myasthenic globulin, to determine that dilution of fluorescein tagged myasthenic globulin which would still be capable of staining sections in the face of a competitive block of binding sites with untagged globulin. Whereas a 1:5 dilution of fluorescein conjugated myasthenic globulin produced bright fluorescence in muscle sections *not* previously treated with untagged, undiluted myasthenic globulin, use of this dilution on sections previously treated with unconjugated, undiluted myasthenic globulin, produced little or no fluorescence. Dilutions 1:4 and 1:2.5 of the

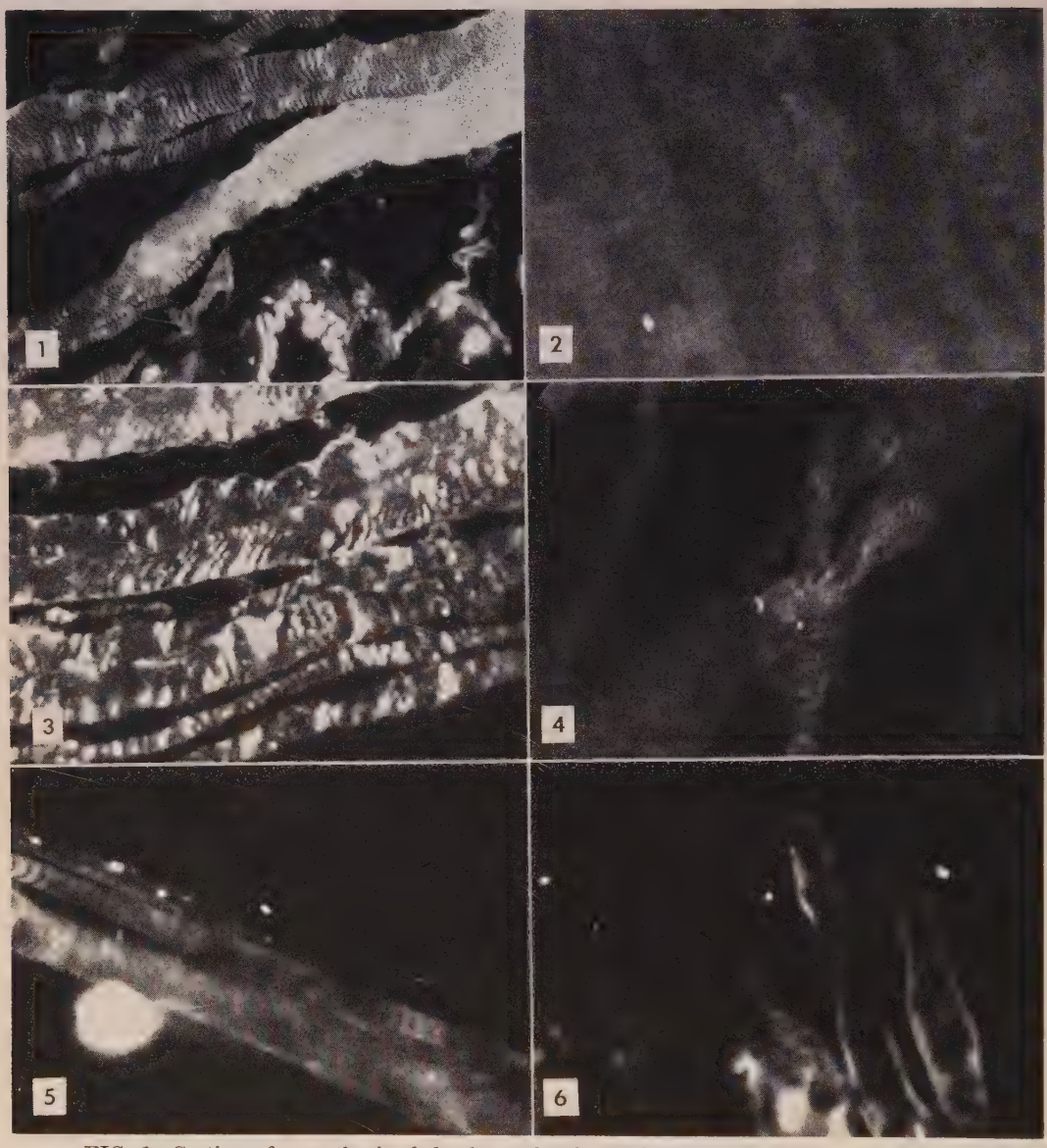

FIG. 1. Section of myasthenic skeletal muscle biopsy A. stained with fluorescein labelled globulin prepared from a pool of 10 myasthenic serums. Note delicate fluorescent striations. Haziness of fluorescence in mid-diagonal fiber, due to the fact that it is in part out of focus. Wavy fluorescence in lower middle field represents autofluorescence in tunica elastica of an arteriole. Magnification 350 ×.

FIG. 2. Section of myasthenic skeletal muscle biopsy B. treated with fluorescein labelled normal human globulin. No fluorescence. Magnification 350 ×.

FIG. 3. Section of myasthenic skeletal muscle biopsy A. treated successively with untagged, undiluted myasthenic globulin, guinea pig complement, and fluorescein labelled rabbit anti-guinea pig complement. Note disruption of fluorescent striations. Magnification 350 ×.

FIG. 4. Section of myasthenic skeletal muscle biopsy A. treated successively with undiluted untagged myasthenic globulin, heat inactivated guinea pig complement, and fluorescein tagged rabbit anti-guinea pig complement. Note that fluorescence, though present, is markedly reduced when compared with section in Fig. 3. Magnification 350 ×.

FIG. 5. Section of myasthenic muscle B. treated with serum of a patient with paroxysmal myoglobinuria, then successively with guinea pig complement and fluorescein tagged rabbit-anti-guinea pig complement. Large fluorescent spot in lower left field represents bubble in

myasthenic globulin, fluorescein tagged showed very appreciable diminution of fluorescence in striations when sections had been previously treated with untagged, undiluted myasthenic globulin. It was concluded, therefore, that untagged myasthenic globulin could competitively inhibit the adherence of fluorescein conjugated myasthenic globulin fraction to skeletal muscle striations. An interesting, as yet unexplained observation was that when skeletal muscle sections, were successively treated with untagged, undiluted normal globulin (15 minutes) and then with fluorescein tagged myasthenic globulin, undiluted or in dilutions to 1:5, fluorescent striations appeared somewhat crisper to the eye than when fluorescein tagged myasthenic globulin was used alone.

A limited series of experiments has been completed in an effort to determine the effect of individual myasthenic serums in blocking the staining produced by the fluorescein conjugated myasthenic globulin fraction. Included in this study were 10 individual myasthenic serums which also contributed to the myasthenic serum pool, and 3 additional myasthenic serums taken from patients with disease of less than one year's duration. No effort has been made rigidly to quantitate these observations, but an acceptable generalization can be made that all muscle sections treated with these selected myasthenic serums for from 15 to 45 minutes evidenced appreciable diminution in intensity of fluorescent staining when sections were treated subsequently with fluorescein conjugated myasthenic globulin fraction 1:4 for 25 minutes; compared with sections treated with fluorescein tagged myasthenic globulin 1:4, alone. This generalization assumes more significance in view of the fact that prior treatment of skeletal muscle sections with 6 normal serums resulted in a *sharper* delineation of fluorescent striations produced by subsequent treatment of sections with fluorescein conjugated myasthenic globulin, 1:4.

In view of the apparent demonstration of a muscle binding globulin exclusive to the myasthenic globulin pool, and the observations of Nastuk, *et al.*, with respect to alterations in serum complement activity in myasthenia gravis(2,3), a test was made of the ability of the untagged myasthenic globulin fraction, when bound to skeletal muscle, to fix guinea pig complement. The technic of Klein and Burkholder(8) for demonstration of complement fixation by fluorescence microscopy was used. Sections of human skeletal muscle were treated successively with: 1. untagged, undiluted myasthenic globulin, 2. guinea pig complement in appropriate dilution, and 3. fluorescein conjugated rabbit anti-guinea pig complement. Results, together with control observations, are presented in Table I. Experiments were carried out in identical fashion with sections from 2 myasthenic muscle biopsies A. and B., and one normal muscle biopsy, F. The localization of guinea pig complement by this immunofluorescence technic is similar to that of the fluorescein conjugated myasthenic globulin, but there are obvious differences. Whereas the fluorescein conjugated myasthenic globulin alone localizes in striations in a rather discrete manner, complement localization, in sections treated with untagged myasthenic globulin, as demonstrated by the use of fluorescein conjugated rabbit anti-guinea pig complement, results in a fluorescent pattern of parts or particles of alternate skeletal muscle striations. Striations and sarcolemma in the latter sections also seem disrupted. On inspection with ultraviolet dark field illumination, *none* of the control sections (Table I) treated with guinea pig complement evidenced marked disruption of muscle fiber membranes. It is conceivable that this disruption might be due to cytolytic activity of complement *fixed* by the myasthenic globulin fraction. This awaits further study.

Finally, we screened 31 myasthenic serums from 30 individual patients, 11 individual normal serums, and 5 serums from other generalized myopathies; these were tested for their

glycerin. Note minimal fluorescence in some striations. Magnification 350 ×.

FIG. 6. Section of normal human skeletal muscle biopsy F treated with serum from a patient with acute dermatomyositis, and successively with guinea pig complement and rabbit anti-guinea pig complement, fluorescein tagged. Note sarcolemmal fluorescence. Magnification 350×.

TABLE I. Histoserological Demonstration of Fixation of Guinea Pig Complement to Sections of Human Skeletal Muscle Previously Treated with Serum Globulin from Patients with Myasthenia Gravis.

Successive treatment of muscle sections with:						
1.	Wash, min.	2.	Wash, min.	3.	Wash, min.	Fluorescence
Buffer (15 min.)	30	Buffer (1 hr)	30	Rab. anti-G.P. comp. "F" (1 hr)	10	None
Idem	30	G. P. comp. 1:30 (1 hr)	30	*Idem*	10	"
Myasthenic glob. untagged, undiluted (15 min)	30	*Idem*	30	"	10	Alternating fluorescent striations (Fig. 3)
Idem	30	Heat inactivated (56°C, 30 min.) G. P. comp. 1:30 (1 hr)	30	"	10	None in most sections. Few faint striations in others (Fig. 4)
"	30	Rab. anti-G.P. comp. "F" (1 hr)	10	—	—	None
Normal glob. untagged, undiluted (15 min.)	30	G. P. comp. 1:30 (1 hr)	30	Rab. anti-G.P. comp. "F" (1 hr)	10	"
Idem	30	Heat inactivated (56°C, 30 min.) G. P. comp. 1:30 (1 hr)	30	*Idem*	10	"
"	30	Rab. anti-G.P. comp. "F" (1 hr)	10	—	—	"

ability to fix complement in relation to skeletal muscle. Successive treatment of muscle with the whole serum in question, guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement was carried out, and the following observed: 1. Sections treated with serums from 8 of 10 myasthenics included in the original myasthenic serum pool, when successively treated with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced fluorescent striations. Skeletal muscle sections exposed to 2 remaining serums included in the original pool failed to fix guinea pig complement; hence, no fluorescence was seen. 2. Sections treated in like manner with serums from all of 5 other myasthenics with disease of less than one year's duration, when treated successively with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced fluorescent striations. 3. Not all myasthenic serums gave rise to fluorescent skeletal muscle sections by this technic; 16 serums taken from individual patients without regard to clinical course gave negative or equivocal results. (Subsequent check revealed that 5 of the 16 serums had come from patients with clinically apparent myasthenia gravis of less than 2 years' duration). 4. One serum sample drawn from patient #1 of the myasthenic serum pool, some 4 months post-thymectomy, fixed complement by this technic. 5. *No sections* treated with the *11 normal serums,* then successively with guinea pig complement and flourescein conjugated rabbit anti-guinea pig complement, evidenced fluorescence in striations. 6. One serum from a patient with exacerbating paroxysmal myoglobinuria gave rise to fluorescent striations in a skeletal muscle section following use of guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement (Fig. 5). 7. A section of normal human skeletal muscle treated with serum from a patient with acute dermatomyositis, and successively treated with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced sarcolemmal fluorescence (Fig. 6). 8. Single serum samples from 3 patients with progressive muscular dystrophy, idiopathic acute polymyositis, and myopathy associated with periarteritis nodosa, respectively, yielded negative results by this technic.

Discussion. These preliminary observations are provocative of several questions which await further study. Is this muscle binding, complement-fixing globulin factor peculiar to one or is it a property of several of the serums included in the myasthenic pool? Our data with respect to blocking and complement fixation with individual myasthenic serums on skeletal muscle suggest its presence in 8 of 10 serums comprising the pool. Is what we have demonstrated by fluorescence microscopy a true immunologic phenomenon? Is it a reaction between a specific muscle antigen and an auto-antibody; if this should be the case, what is the nature of the antigen involved? The ability of this system to fix complement suggests an immune phenomenon. The more classical technics, agar diffusion, precipitation methods, agglutination, and test tube complement fixation should be employed to further this study. Does this material, unique to the myasthenic globulin fraction, represent gamma globulin, or possibly alpha or beta globulin? Our globulin preparations contain alpha, beta, and gamma fractions. Further separation of myasthenic pooled globulins into alpha, beta, and gamma components is under way.

Other questions include the origin of the factor peculiar to the myasthenic globulin and its relation, if any, to the pathogenesis of the disease. Is its presence in any way related to the thymic pathology seen in association with myasthenia gravis? What relation do these observations bear to the derangement in serum complement in myasthenia gravis? Is the presence of a complement-fixing, muscle binding globulin in serum a feature of myasthenia gravis exclusively, or can it be seen in other myopathies?

Summary. 1. A muscle binding, complement fixing component has been demonstrated in the crude globulin fraction of a pool of serum from 10 patients with myasthenia gravis of recent onset and progressive character, by means of the immunofluorescence technic. This component could not be demonstrated in a similarly prepared normal serum globulin pool. Untagged myasthenic globulin was shown to block competitively adherence of fluorescein tagged myasthenic globulin to skeletal muscle striations; whereas prior treatment of muscle sections with normal serum globulin intensified staining with fluorescein tagged myasthenic globulin. Individual myasthenia gravis serums included in the pool also blocked staining with fluorescein conjugated myasthenic globulin. Normal serums did not block adherence of the fluorescein tagged myasthenic globulin to skeletal muscle. 2. The myasthenia gravis globulin fraction was shown to fix guinea pig complement to human skeletal muscle, by successive treatment of muscle sections with myasthenic globulin, guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement. Normal serum globulin failed to fix complement by this technic. 3. Thirty-one myasthenia gravis serums were screened, with 11 normal serums, and 5 serums from patients with other generalized myopathies, for their ability to fix guinea pig complement to skeletal muscle. Thirteen myasthenic serums gave unequivocal evidences of complement fixation, using the immunofluorescence technic. No normal serums fixed complement. Serum from one patient with paroxysmal myoglobinuria also fixed complement. Serum from one patient with acute dermatomyositis gave rise to fluorescence in the sarcolemma when layered onto skeletal muscle followed successively by guinea pig complement and fluorescein conjugated rabbit anti-guinea pig complement.

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Fatty Acid Composition of Feces and Fecaliths.* (26052)

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The presence of appendiceal concretions, or fecaliths, is an important factor in the genesis of acute distinctive appendicitis(1,2). Early reports of appendicitis often refer to the presence of a fecalith or a foreign body (3,4). The fact that many fecaliths were mistaken for fruit stones led to the popular misconception that fruit stones were the cause of appendicitis. As early as 1813, the fact that fecaliths contained lipid material was noted by Wegeler(5). The first detailed study of fecalith composition was by Williams(6), who considered that they were formed largely of calcium soaps of palmitic and stearic acids. Maver and Wells(7) found 50% of the dry weight of fecaliths to be soluble in ether or hot amyl alcohol. They concluded that there was a high proportion of lipids present as insoluble soaps. Bowers(2) compared the composition of fecaliths and fecal pellets and concluded that the former were of a specific composition, and were not simply inspissated feces. He suggested that fecaliths were formed in the lumen of the appendix by a deposition of layers upon a central nucleus of undigested material.

Whether the material that goes to form the layers of a fecalith comes from the feces or is excreted by the appendix is not known. However, Williams suggested that fatty acids are excreted by the wall of the appendix in the form of calcium soaps(8). Sperry *et al.* (9,10) showed that fatty acids, largely palmitic and stearic acids, are excreted by the intestinal mucosa in the dog. If the pattern of fatty acids in fecaliths is the same as that of the acids excreted by the intestinal mucosa and different from that of fecal fatty acids, Williams' theory of appendiceal fatty acid excretion would be supported.

The purpose of this study was to compare the relative proportions, in fecalith and fecal lipids, of the 4 fatty acids most abundant in nature and the human body, namely palmitic, stearic, oleic and linoleic acids, to determine the types of lipids present in fecaliths and feces and to make some comparisons.

Materials. A total of 52 fecaliths, stored in 10% formalin solution, were obtained from the collection made by the University Hospital during the past 5 years. Feces were obtained from normal adults, with no symptoms of bowel disturbance, who were on an "average" North American diet.

Methods. Methyl esters of fatty acids were prepared from a petroleum ether extract of 6 groups of fecaliths, and from 5 fecal samples. It was initially assumed from previous reports(6,7,8) that the fatty acids would be present largely in the form of soaps. The samples, therefore, were homogenized and acidified with HCl (pH 1) to liberate free acids. Lipids were extracted with petroleum ether (b.p. 35-45°C) and after removal of the solvent, saponified with excess ethanolic potasium hydroxide. The non-saponifiable material was removed by ether extraction of the hydrolysate, the aqueous phase was acidified and the fatty acids extracted with ether. These acids were converted to methyl esters with methanolic hydrogen chloride. In addition, 3 fresh fecalith samples and 3 samples of feces were extracted with chloroform-methanol (2:1, v/v) for analysis of their total lipids.

It is appreciated that all the lipids would not have been extracted from the first group of fecalith and fecal samples using petroleum ether. However detailed analysis of the fatty acid content of the various fractions of a total lipid extract has since shown that the relative proportion of the 4 fatty acids under consideration is not significantly altered by excluding the lipids insoluble in petroleum ether. This analysis will be reported later.

Gas-liquid chromatography (GLC) was carried out with a 6 ft. x ¼ in. I.D., U-

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TABLE I. Relative Proportions of 4 Major Naturally Occurring Fatty Acids in Fecaliths and Feces as Determined by GLC and Expressed as % of Their Total Area.

Acids	Fecalith samples							Fecal samples					
	1	2	3	4	5	6	Mean	1	2	3	4	5	Mean
Palmitic	39	42	36	44	45	43	41	26	26	29	27	17	25
Stearic	53	46	55	47	45	41	48	31	26	33	26	44	32
Oleic	8	12	9	9	10	16	11	35	36	31	33	33	33.5
Linoleic	—	—	—	—	—	—	—	8	12	7	14	6	9.5

shaped copper column having Craig polyester,‡ as stationary phase, coated on acid-washed Chromasorb W (25:75 by weight). The column was operated at 212°C and helium was used as the carrier gas flowing at 130 ml/min at a head presure of 22 p.s.i. Detection was by means of a thermal conductivity cell.

Thin-layer chromatography. This elegant method developed by Stahl(11,12) and applied to analysis of lipids by Mangold and Malins(13,14) was used to study the total lipid extract from fecaliths and feces. Samples of 0.01 to 1.0 mg, in solution, were spotted along one edge of the plate and separated by ascending elution. A suitable solvent for separation of these samples was found to be a mixture of petroleum ether (b.p. 35-45°C), diethyl ether and acetic acid (80:20:1, v/v/v) and spots were made visible by spraying the plates with 50% sulphuric acid and heating to char the organic material (16). The fluorescence of many of the spots or bands under ultraviolet light enabled their positions to be determined without their destruction so that individual fractions could be scraped off the plate, extracted from the adsorbent and weighed. To obtain large enough amounts of each component for weighing, 20 to 25 spots, each containing 1-2 mg of sample, were run on a single plate and each whole row of spots (or band) corresponding to a component was then scraped off.

Results. Gas-liquid chromatographic analysis of methyl esters derived from fecalith lipids demonstrated the presence of as many as 30 fatty acid esters in the range of C-10 to C-24. Tentative identification and possible significance of the other fatty acids present will be reported later.

‡ Butanediol-pentaerythritol succinate polyester. (Obtained from Wilkens Instrument & Research, Inc., California).

The proportions of the 4 major fatty acids were calculated as % of their total area under the GLC curves, other minor components being excluded from the calculations (Table I).

From the relative proportions of these 4 fatty acids in fecaliths and feces, it is obvious that fecalith lipids differ markedly from fecal lipids in containing no apparent linoleic acid and a much smaller proportion of oleic acid.

Chloroform-methanol extraction of fecaliths removed 20-25% of the dry weight of the samples. Acidification of the residue with HCl to liberate fatty acids from any insoluble soaps enabled a further 3-4% of the dry weight to be dissolved in chloroform-methanol. This fraction, however, did not consist of fatty acids because it did not migrate like a standard fatty acid sample on a thin-layer chromatogram, but remained at the starting point. It seems, therefore, that none of the lipids in fecaliths exist in the form of soaps.

Thin-layer chromatography of the total lipid extract resulted in separation of 7 distinct lipid classes. These types were compared with known standards run simultaneously on the same plate (Fig. 1). Additional information about the nature of the components of individual spots was obtained by watching the development of colors on heating, after spraying with sulphuric acid(16). For example, cholesterol and cholesteryl esters are among the first to show color, the spots being a characteristic bright pink. Thus, band 7 of the fecalith lipids (Fig. 1) showed early coloration coincident with the cholesteryl ester standard but later was covered with a darker brown spot due to another component, as yet unidentified.

To conduct a quantitative analysis of the lipids 25-50 mg of these were spotted on a single plate and separated. After localization

FIG. 1. Thin-layer chromatogram of fecalith and fecal lipids and standards. Plate was eluted with a mixture of ethyl ether, petroleum ether and acetic acid (20:80:1, v/v/v), the spots developed by charring after spraying with 50% sulphuric acid and reproduction made by photo-copying. Samples were: A, monoolein; B, 1,2- and 1,3-diolein; C, triolein; D, cholesteryl palmitate; E, oleic acid; F, fecal lipids; G, fecalith lipids; H, cholesterol; I, 14-hydroxystearic acid; J, 2-hydroxystearic acid; K, selachyl alcohol; L, yeast lecithin.

under ultraviolet light the individual bands were extracted from the adsorbent and weighed. Table II shows the proportion of the lipids found in the bands from feces and from fecaliths. Approximate figures only are given, as the relative inaccuracy of this technic is appreciated. Tentative identification of some of the lipid classes was made from comparison with standards. The presence of cholesterol in bands 3 and 7 and of phosphorous in band 1 was verified chemically.

Discussion. Fecaliths have been shown to have a lipid content of 20-25% of their dry weight, the remainder being inorganic calcium salts plus undigested fecal debris. Contrary to previous opinion, the lipid fraction of fecaliths does not appear to exist in the form of soaps but approximately one-third is present as free fatty acids. Compared with the lipids from feces, fecalith total lipids contain much less triglyceride and more free fatty acids and the component fatty acid composition is significantly different from that of feces. These observations suggest that fecalith lipids are not simply a quantitative deposition of fecal lipids.

TABLE II. Proportion of Total Lipid Extract, by Weight, Recovered from the Bands Separated by Thin-Layer Chromatography.

Band No.	Identification	Fecaliths %	Feces %
7	Cholesteryl esters and unknown	10-25	5-10
6	Triglycerides	2- 5	10-15
5	Free fatty acids	25-36	12-20
4	Unknown	24-29	22-32
3	Cholesterol and unknown	4-10	10-15
2	Unknown	3- 8	10-15
1	Phospholipids and unknown	5-10	8-10
% of original sample recovered		ca. 95	ca. 90

If it is assumed that fecaliths form within the appendix, then the possible sources of the fecalith lipids are either the secretions of the appendix or the feces that pass in and out of the appendix. If deposition from feces occurs, there must either be a selective deposition of certain lipids, particularly fatty acids, or there must be some alteration of the lipids when once deposited.

Williams' theory of appendiceal secretion of lipids would appear to be substantiated by the finding of a predominance of palmitic and stearic acids, but no convincing proof of the secretion of lipids by the human appendix has been found. Indeed the appendiceal secretion of the rabbit appendix has been found to contain no significant lipids even after forced fat feeding(17). There may be, however, considerable difference in function of the appendix in man and the rabbit.

Differential deposition of free fatty acids can occur only in an acid medium. The pH of obstructed human appendices has been shown to be in the range 6.0-8.3(18). The laminated appearance of many, but not all, fecaliths suggests an alternating deposition of lipids and inorganic calcium salts, possibly resulting from variations between acid and alkaline conditions.

The alteration of deposited lipids could result from bacterial action and the large number of peaks obtained by GLC analysis of the methyl esters derived from fecaliths, many of which are due to esters of branched chain acids, suggest that such bacterial action in the appendix may be important. Chipault *et al.* (19) have shown that incubation of triolein with a culture of stool resulted in conversion of unsaturated to saturated acids. The small proportion of unsaturated acids in fecalith

lipids may be the result of similar bacterial action within the appendix. Further information about the nature and fate of lipids secreted into the bowel should help to determine the reasons for the characteristic and relatively constant lipid pattern of the fecalith.

Summary. The relative proportions of palmitic, stearic, oleic, and linoleic acids in lipids extracted from fecaliths and feces are compared by means of gas-liquid chromatography. Fecaliths, in contrast to feces, contain little oleic acid and no apparent linoleic acid. Thin-layer chromatography was used to determine the types of lipids found in fecaliths and feces. The former contain less triglyceride and more free fatty acids than the latter. Fatty acid soaps were not found in fecaliths.

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Electrocardiogram in Hamsters after Large Fat Meals.* (26053)

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It has been shown in hamsters that the red blood cells aggregate and the circulation slows after large fat meals(1,2). Availability of oxygen in cerebral tissues is also significantly decreased(3) and convulsions occur(4) after meals of fat. These changes are either absent or much less marked after meals of oil. This paper reports changes in the electrocardiograms which also occur after fat, but not after oil meals.

Material and methods. Hamsters weighing from 80 to 120 g were tube fed butterfat as cream, or oil and synthetic fat mixtures emulsified in skim milk under very light ether anesthesia. Amounts of the lipid meals varied from 1.6 to 10.0 g/kg body weight. The volume of each feeding was the same, 3 ml/100 g body weight; concentration of the lipid in the meals varied. Standard 3 lead electrocardiograms were determined 0, 3, 6, 9, 24, 48, and 72 hours after each feeding. The animals were restrained by leg ties for recording of the E.K.G.s. E.K.G. potentials were amplified by a Tektronic polygraph and recorded by an Offner dynograph.

Results. The electrocardiographic changes after fat meals consisted of elongation of QT and ST intervals. The QT interval was measured from the beginning of the QRS complex to the end of the T wave (where the T wave returns to the isoelectric line). The ST segment was measured from the end of the QRS complex to the beginning rise of the T wave. In 55 animals before lipid feeding RR, QT, and ST intervals were measured in the stand-

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FIG. 1. Electrocardiogram of hamster after cream meal of 10 g butterfat/kg body wt showing elongation of QT interval and ST segment, and changes produced by inj. of calcium.

FIG. 2. Relationship of QT interval to RR interval in 55 non-fat fed animals, and in 9 cream fat fed animals. Only the one longest QT interval in each animal experiment was recorded after fat feeding.

ard 2 lead. In all 55, ST intervals were too short to be measured, or were non-existent depending upon the shape of the QRS complex. The QT interval varied from 0.08 to 0.12 seconds with a tendency for the QT interval to vary directly with length of the RR interval up to a RR interval of about 0.15 seconds. Further increase in RR interval did not increase the QT interval in normal animals (Fig. 2).

After feeding 4 g cream fat/kg body weight the QT interval did not exceed the normal limit of 0.12 seconds in any one of 9 animals up to 72 hours, and the ST segment was still too short to be measured. After feedings of cod liver oil (10.0 g/kg in 4 animals), olive oil (8 g/kg in 6 animals), safflower oil (8 g/kg in 6 animals), and hydrogenated safflower oil with iodine number of 99 (8 g/kg in 6 animals) no abnormally long QT or ST intervals were found when similarly measured.

After feeding of 8 g of cream fat/kg body weight, QT interval in 2 of the 8 animals was prolonged up to 0.14 and 0.17 seconds 3 to 9 hours after feeding. The ST segment was prolonged in 7 of the 8 hamsters to from 0.01 to 0.08 seconds (Fig. 1). After 10 g cream fat/kg the QT interval was prolonged in 7 of 9 animals (Fig. 2), and ST segment was prolonged to from 0.02 to 0.1 seconds in all animals. Prolongation of ST segments was evident 3 hours after feeding in all animals. Three hours later the QT interval was measurably elongated. Elongation of these intervals continued for from 3 to 9 hours in most animals, and for 72 hours in one animal. Six animals were fed a synthetic formula containing olive oil 30%, tributyrin 10%, and tricaprin 60% in 8 g/kg doses. This formula has been shown to produce frequent convulsions in hamsters(5). In one animal the QT interval was prolonged and in 2 others the ST interval was prolonged. In all instances the changes in QT interval were similar to those described by Kleinfeld and Gross(6) in rabbits after injections of EDTA.

Injections of 30 mg of calcium gluconate into 5 animals with prolonged QT intervals shortened the interval to or near to normal for several hours in 4 of 5 animals (Fig. 1). The ST segment was altered similarly in the same 4 animals. Injections of 10 g potassium chloride in fat fed animals altered the amplitude and/or form of the QRS complex and T wave without shortening the QT or ST segments in 2 of 3 animals.

Discussion. From other studies(3,5) it is evident that the incidence and magnitude of the changes in QT and ST interval varies directly with decreases in available oxygen in the tissues. It seems unlikely, however, that hypoxia alone is the cause of the QT and ST changes, since these intervals were rather quickly returned nearly to, or to normal by injections of calcium gluconate. It seems more likely that the metabolism of large

amounts of lipid contributed to the E.K.G. changes by binding the freely available calcium. The possibility exists that the cations in the tisues and blood were utilized to neutralize the free fatty acids liberated by hydrolysis of neutral fats.

We have shown that convulsions occur in hamsters after fat meals, but not after similarly sized meals of highly unsaturated oils such as cod liver oil and safflower oil(4,5). The mechanisms of the seizures is not known, but is thought to be related to alterations in the circulation(1,2) and reduction in availability of oxygen in the brain(3) which occur after fat feedings. It is postulated that the permeability of brain capillaries is increased and free fatty acids, normally excluded from the brain, pass the blood-brain barrier in toxic or irritant amounts. No direct relationship can at present be established between the mechanisms of the seizures and E.K.G. changes. The fact that the changes noted above occur after fat meals and not after oil meals indicate that the metabolism of saturated fatty acids is significantly different, quantitatively or qualitatively, from the metabolism of unsaturated fatty acids.

Summary. Large cream and oil meals were fed to hamsters. At intervals after feeding electrocardiograms were made. After the fat meals prolongation of the QT interval, especially its ST segment occurred. These changes were absent after oil meals.

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Gonadotrophin Inhibiting and Anti-Fecundity Effects of Chloramiphene.* (26054)

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Modification of the chemical structure of chlorotrianisene (TACE®)(1) has led to a varied spectrum of biological activities. The specific biological effects produced by these modified substances are: (a) inhibition of cholesterol synthesis by triparanol (MER/29®)(2), and (b) estrogen antagonism by ethamoxytriphetol (MER-25)(3). The present paper pertains to a new structurally related compound, chloramiphene (1-[*p*-*β*-diethylaminoethoxy) phenyl] -1,2-diphenyl-2-chloroethylene).‖ Its major biological activity refers to pituitary gonadotrophin inhibition and anti-fecundity.

Materials and methods. Chloramiphene was tested as the citrate salt. It was administered in suspension in an olive oil vehicle unless otherwise indicated. Control animals received the vehicle alone. Rats utilized were of Sprague-Dawley strain, and were given tap water and stock laboratory diet (Rockland) *ad libitum. Pituitary gonadotrophin inhibition. Intact immature male rats.* Rats of approximately 70 g were divided into groups of 5 and were given chloramiphene in doses ranging from 0.3 to 70.0 mg/kg/day, by once

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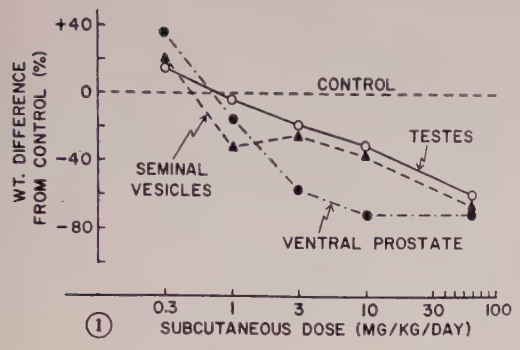
‖ One of a series of structurally related compounds, synthesized by Allen, R. E., Feil, V. J., and Palopoli, F. P.

daily subcutaneous injection for 10 days. Autopsies were performed on the day after last injection. Organs were removed and weighed. *Intact mature male rats.* Rats weighing 310 to 350 g were divided into 6 groups of 5 animals each. Three groups received 1.0 mg/kg/day chloramiphene subcutaneously. At the end of each 5, 10 and 20 days, one group of control and one group of treated animals were sacrificed and autopsied. Testes, seminal vesicles, ventral prostate, and pituitary were removed and weighed. *Intact immature female rats.* Chloramiphene was given subcutaneously at 1.0 mg/kg/day. One control and one treated group (5/group) were autopsied after each 10 and 20 days of treatment. Ovaries, uteri, and pituitaries were removed and weighed. *Parabiotic rats.* Litter-mate female rats, 30-32 days of age, were parabiotically joined(4). One parabiont was ovariectomized. Chloramiphene was given to the castrate partner at doses ranging from 0.001 to 1.0 mg/castrate parabiont/day for 10 days. Each dose level was tested in 3 to 5 parabiotic pairs. Effectiveness was evaluated by both subcutaneous and oral routes. Ovaries of the intact parabiont and uteri of both animals were removed and weighed at time of autopsy. *Gonadotrophin content of pituitaries.* Pituitaries were removed from a group of 5 donor control animals and a group of 5 donor animals treated for 20 days with chloramiphene at 1.0 mg/kg/day. Pituitaries were pooled according to group, macerated and homogenized in isotonic saline. They were then administered subcutaneously to 22-day-old female recipient rats, once daily for 3 days in a dose equivalent to 1 pituitary/rat/3-day period. Pregnant mare serum§ (PMS, Gonadogen®) was used as a control standard. On the day after last injection the rats were sacrificed. Uteri and ovaries were removed and weighed. *Absence of antagonism to exogenous gonadotrophin.* Chloramiphene (3 mg/kg/day) was administered subcutaneously for 4 days to 22-day-old female rats (7/group). Exogenous gonadotrophin§ (PMS) was administered at a separate injection site for each of the last 3 days of chloramiphene treatment. On the day after last injection the rats were autopsied. Ovaries and uteri were removed and weighed. *Interruption of estrous cycles of rats.* Young, adult female rats were divided into control and experimental groups of 4 to 7 animals each. Daily vaginal smears were studied for at least 2 full cycles before start of drug. Chloramiphene was given either subcutaneously or orally at doses ranging from 0.01 to 3.0 mg/kg/day for 6 or 8 days. Vaginal smears were taken once daily. *Anti-fecundity activity.* Young, adult, female rats were divided into control and experimental groups of 3 to 7 animals per group. Chloramiphene was given to female rats, either subcutaneously or orally in doses ranging from 0.01 to 3.0 mg/kg/day. Fecundity was determined for the period during drug administration, the period immediately after stopping the drug, and period after return of normal sex cycling after stopping treatment. Treatment was daily for 6 days for the last experimental approach, 8 days for the second, and from 8 days before cohabitation and then during cohabitation through the second day after observation of sperm cells in the vagina, for the first test approach. Males were rotated during cohabitation.

Results. In *immature male rats* chloramiphene in doses from 1 mg/kg/day and greater yielded lower relative weights of testes, seminal vesicles, and ventral prostates (Fig. 1). Degree of lowering was dose dependent. Ventral prostates were lowered more than either seminal vesicles or testes. Rate of body weight gain was not altered at doses below 3 mg/kg/day. Increasing the dose above this amount caused a progressive decrease in rate of weight gain. Rats receiving the highest dose of 70 mg/kg/day gained 33 g in weight while control animals gained 65 g. In *mature male rats,* chloramiphene-treated rats had lower ventral prostate and seminal vesicle weights after 20 days of treatment with 1 mg/kg/day (Table I). These organs were about three-fourths of control weight. No difference was observed at 10 days. Pituitary weights in these mature male animals treated for 10 or 20 days were not altered by chloramiphene.

§ PMS dose: Total dose of 20 I.U./rat given in 3 days, e.g. 6.6. I.U./rat/day.



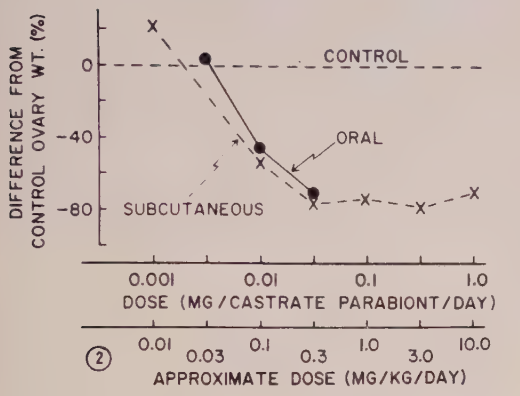


FIG. 1. Effects of chloramiphene on weights of testes, seminal vesicles, and ventral prostates of immature rats. Treatment was by subcut. inj. for 10 days.

FIG. 2. Gonadotrophin inhibiting activity of orally and subcut. administered chloramiphene to immature parabiotic littermate female rats (1 castrate : 1 intact). Ovarian wt was the index of response. Chloramiphene was given for 10 days to the castrate parabiont. Approximate dose is based on wt of castrate parabiont.

In intact immature female rats subcutaneous treatment at 1 mg/kg/day for 10 days produced a lowering of mean relative uterine weight. It was 59% of mean control weight (115 mg/100 g body weight). Ovarian weight was not altered (27 mg/100 g body weight). Subcutaneous administration at the same dose for 20 days resulted in a lowering of both uterine and ovarian weights to 62% and 29% below the controls, respectively. The same degree of lowering was obtained by 10-day oral administration of 2 mg/kg/day (uterus and ovary, 44% and 21% below controls, respectively). After 10 days and 20 days of subcutaneous treatment, mean relative pituitary weights were 13% and 19%, respectively, below control weight. The oral dose had no effect (2% above controls). In *parabiotic rats,* chloramiphene at a dose of 0.01 mg/parabiont/day (approximately 0.1 mg/kg/day on the basis of body weight of castrate parabiont) resulted in ovarian weights that were 50% of control (Fig. 2). Doses of 0.3 mg/kg/day reduced ovarian weight to a plateau of response approximating 20% of control. Oral and subcutaneous routes of administration were equally effective.

The *gonadotrophin content of pituitaries* from adult male rats was examined after 20 days of treatment with chloramiphene at 1 mg/kg/day. Pituitaries from control and treated donor rats produced comparable increases in ovarian (2.8- and 2.9-fold respectively) and uterine (3.5- and 3.7-fold) weights of the immature recipient test rats. Thus, the pituitary gonadotrophin content was not altered by treatment.

Chloramiphene *failed to block exogenous gonadotrophin* (PMS). When chloramiphene was given at 3 mg/kg/day along with PMS at 20 I.U./rat, mean weight of the ovaries was 96 mg. Rats receiving only PMS had a mean ovarian weight of 83 mg. The increased uterine weight (125 mg) induced by PMS treatment alone was, however, reduced to 78

TABLE I. Effect of Chloramiphene (1.0 mg/kg/Day Subcutaneously Once Daily) on Organ Weights of Mature, Intact, Male Rats.

Group	Length of treatment (days)	Body wt*		Relative organ wt (mg/100 g body wt)*			
		Initial (g)	Final (g)	Testes	Seminal vesicles	Ventral prostate	Pituitary
Control	5	355 ± 9	373 ± 10	830 ± 51	94 ± 6	130 ± 6	2.7 ±.11
Treated	5	341 ± 12	344 ± 15	980 ± 31	84 ± 2	120 ± 6	2.5 ±.10
Control	10	342 ± 10	356 ± 12	900 ± 46	95 ±11	120 ±11	2.8 ±.12
Treated	10	343 ± 12	338 ± 9	950 ± 37	83 ± 4	120 ± 9	2.6 ±.05
Control	20	329 ± 9	349 ± 9	910 ± 21	92 ± 7	120 ± 8	2.8 ±.15
Treated	20	312 ± 7	303 ± 6	1060 ± 48†	68 ± 6†	94 ± 7†	2.7 ±.15

* Mean ± S.E.

† $.05 > P > .02$ by t test(6).

TABLE II. Interruption of Estrous Cycles by Daily Administrations of Chloramiphene to Young Adult Female Rats.

Dose (mg /kg/day)	Route	Vehicle	Rats with interrupted cycles / No. of rats
Control	subcut.	oil	0/5
.3	"	"	5/5
Control	oral	"	0/15
.01	"	"	0/4
.1	"	"	4/4
.3	"	water	7/7
1.0	"	"	7/7
3.0	"	oil	5/5

mg by concurrent chloramiphene treatment. At this dose, chloramiphene alone doubled uterine weight from 29 to 59 mg, but had no effect on mean ovarian weight (17 mg).

Chloramiphene doses of 0.1 mg/kg/day and greater *interrupted the estrous cycle* in young adult female rats (Table II). The lower dose of 0.01 mg/kg/day was not effective. Interruption of the sex cycle at 0.1 mg/kg/day was evident after second or third administration of chloramiphene. In this experiment first day of treatment was coincident with metestrus. Cells found in the vaginal smear during the cessation of cycling appeared to be characteristic of incomplete diestrus. The smear was a mixture of mostly leucocytes with only a few epithelial and cornified epithelial cells.

Anti-fecundity activity of chloramiphene was observed in female rats receiving during cohabitation, doses of 0.3 mg/kg/day subcutaneously or 3.0 mg/kg/day orally. None of the 10 treated animals became pregnant whereas 8 of 10 control animals became pregnant. Five orally treated and 3 of 5 subcutaneously treated animals copulated during period of treatment. The 2 non-copulating females did copulate after treatment was stopped. Average time from initial cohabitation to copulation was 4 days. This was equivalent to the time observed in control animals. When treated female rats were started in cohabitation with the male on the day after oral treatment was stopped with either 0.3 or 1.0 mg/kg/day, 6 of 7, and 7 of 7 rats, respectively, became pregnant.

Mean number of days from start of cohabitation to first day of pregnancy was 4.6 days for the control group, 14.8 days after 0.3 mg/kg/day, and 17.6 days after 1 mg/kg/day. In a separate experiment, female rats (3 or 4/group) were treated at 0.01 mg/kg/day or 0.1 mg/kg/day and then allowed to return to normal cycling. They were then cohabitated with males. All these prior-treated animals were fertile.

Discussion. Chloramiphene produced a dose dependent reduction in weight of reproductive organs of intact rats: ovaries and uteri in females, and testes, ventral prostates and seminal vesicles in males. It prevented the ovarian hypertrophy induced by joining castrate and intact rats in parabiosis. Such results are indicative of pituitary gonadotrophin inhibition. Chloramiphene, however, did not change pituitary gonadotrophin content and produced no change or possibly a slight decrease in pituitary weight. Estradiol, on the other hand, produces pituitary hypertrophy(5).

Administration of chloramiphene to mature female rats interrupted normal estrous cycles and produced a loss in fecundity. Both these effects were reversible after drug administration was stopped.

The 3.0 mg/kg/day dose of chloramiphene to immature female rats gave a doubling of uterine weight indicative of weak estrogen-like activity. This level of chloramiphene antagonized the indirect uterotrophic effect of PMS which suggests an anti-estrogen action of chloramiphene. Thus, the vaginal smear picture, the mild uterotrophic effect and antagonism to the uterine weight increase, indirectly induced by PMS, indicate that chloramiphene has secondary mild or atypical estrogen-like and estrogen-antagonizing effects. Pituitary inhibition, however, was the biological effect observed at the lowest dose: *e.g.*, 0.1 mg/kg/day prevented ovarian hypertrophy in immature parabiotic rats and interrupted estrous cycle in mature female rats.

Summary. Chloramiphene (1-[*p*-*β*-diethylaminoethoxy) phenyl] -1, 2-diphenyl-2-chloroethylene) is a pituitary gonadotrophin inhibitor. In immature litter-mate female parabiotic rats (1 castrate: 1 intact), 0.1 mg/kg/day inhibited ovarian weight gain by 50%. Larger doses yielded greater inhibition.

It was equally active by oral and subcutaneous routes. Doses of 0.1 mg/kg/day and greater interrupted the estrous cycles of young mature female rats. Treatment during cohabitation produced a loss in fecundity which was reversible after cessation of treatment. Pituitaries of chloramiphene-treated rats had approximately the same weight and gonadotrophin content as control rats. Chloramiphene did not inhibit exogenously administered gonadotrophin.

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Effect of Reserpine on Pituitary-Gonadal Axis.* (26055)

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Interference of tranquilizing agents, particularly chlorpromazine and other phenothiazine derivatives, with adrenal, thyroid and gonadal functions has been reported by several investigators(1-5). Most authors ascribe this interference to action of the drug on the hypothalamus. Since the hypotensive and tranquilizing effect of reserpine is presumed to result from its action on the diencephalon, and particularly on the hypothalamic region (6-7), reserpine might be expected to interfere with the normal functioning of the endocrine system.

Reserpine was reported to interfere with estrous cycles in mice(8), ovulation and menstruation in monkeys(9), prolactin secretion and discharge in rats, rabbits and women (10-12), oxytocin and prolactin secretion in lactating rats(13), and to effect recession of exophthalmus(14).

In this paper we wish to report on the effect of reserpine on the pituitary-gonadal axis.

Materials and methods. Reserpine stock solution was prepared according to the method described in Martindale's Extra Pharmacopoea 1959, and diluted to the required concentrations. Experiments were carried out on a total of 200 male and female infantile and adult albino rats and 10 male pigeons of the Columba strain. Vaginal smears and gonadotropin determinations were performed on 8 female patients.

The following aspects of the effect of reserpine on the pituitary-gonadal axis were studied: A. Vaginal pattern in infantile rats, adult rats and women; B. Ovarian and uterine development in infantile and adult rats; C. Testicular development in infantile male rats, adult male rats and adult male pigeons; D. Gonadotropin content in urine of menopausal women.

Results. A. Vaginal Pattern. 1. *Infantile female rats.* Twenty rats were injected subcutaneously with 0.05 mg/kg reserpine and another 20 rats with 0.2 mg/kg; 20 controls received the vehicle. In 10% of controls, vaginal opening was complete at age of 35 days, and in 50% at age of 44 days. After this date positive vaginal smears in control animals varied between 20-50%. At dose level of 0.05 mg/kg the stage of vaginal opening and incidence of positive smears closely resembled the pattern observed among the controls (see above). At dose level of 0.2 mg/kg a delay in vaginal opening was ob-

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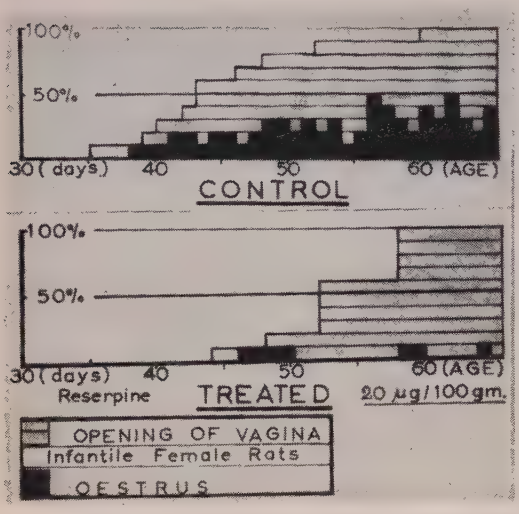

FIG. 1. Effect of reserpine on vaginal opening and estrus in infantile female rats during 40 days' treatment.

served. No opening occurred by the 35th day, there were only 10% openings on the 44th day and 50% on the 52nd day. Daily estrous incidence sank to 10% (Fig. 1).

2. *Adult female rats*: Ten rats which showed normal and regular estrous cycles of 4-5 days were injected subcutaneously with 0.2 mg/kg reserpine daily for 2 successive periods of 15 days each, at an interval of 25 days. Reserpine treatment resulted in a state of diestrus after 3-5 days of treatment, which continued for 3-5 days after withdrawal (Fig. 2).

3. *Women*: Ovarian activity of 3 female patients, 30-40 years old, who received daily reserpine treatment (5-15 mg/d) over a period of 20-60 days, was examined by vaginal smears. They showed successively decreasing estrogen activity throughout treatment.

B. Ovarian and uterine development. 1. *Infantile female rats*: Two groups of 10 rats received subcutaneously daily 0.1 mg/kg and 0.25 mg/kg body weight reserpine, respectively, for 30 days; a third group of 10 rats served as control. At the end of this treatment all animals were sacrificed and their ovaries and uteri were dissected and weighed, showing ovarian weights of 30-36 mg in the first group (0.1 mg/kg reserpine daily) and 8-13 mg in the second group (0.25 mg/kg), as compared with 42-50 mg in the controls. Uterine development was similarly affected, the respective figures being 185-220 mg for the first group, 85-110 mg for the second, as against 300-420 mg in the controls.

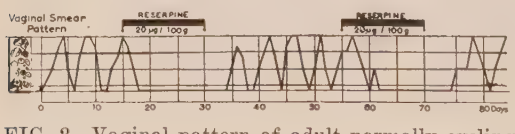

FIG. 2. Vaginal pattern of adult normally cycling female rat during 2 successive treatments with reserpine.

2. *Adult female rats*: Two groups of 10 rats received subcutaneously daily 0.1 mg/kg and 0.2 mg/kg reserpine, respectively, for a period of 40 days; a third group of 10 rats served as control. At the end of this period the animals were sacrificed and ovaries and uteri examined morphologically and histologically. While reserpine caused only slight losses in weights of uterus and ovary, it provoked pronounced morphological and histological changes. No fully mature follicles were found, corpora lutea were highly developed and uteri were atrophic as in lactating rats, suggesting intensified prolactin secretion from the pituitary.

C. Testicular development. 1. *Infantile male rats*: Four groups of 10 rats each were daily injected subcutaneously with 0.01, 0.05, 0.1 and 0.2 mg/kg reserpine, respectively, for 30 days. A fifth group of 10 rats served as control. All animals were examined for testicular descent. While doses of 0.01 mg/kg reserpine daily did not delay testicular descent, an obvious delay was seen at doses from 0.05 mg/kg upwards. The higher the dose, the longer the delay.

2. *Adult male rats*: Four groups of 5 adult male rats each, weighing 200 $\pm$ 15 g, received subcutaneous injections of 0.05, 0.1, 0.25 and 0.5 mg/kg reserpine, respectively, for a period of 15-40 days. Their testes, seminal vesicles and prostates were examined. Low doses of reserpine of 0.05 mg/kg were found to have no effect on morphology or histology of testes, seminal vesicles and prostates. In some, but not all, animals, higher doses caused severe atrophy of the seminal vesicle and prostate and regressive changes in the testes, as seen after hypophysectomy (Fig. 3).

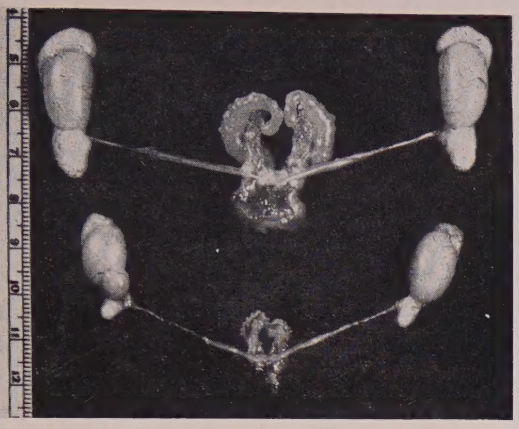

FIG. 3. Accessory sex organs of normal adult rat (above) and reserpine treated adult rat (below).

3. *Adult male pigeons*: Five male pigeons weighing between 250-300 g were given 0.2 mg/kg reserpine. This caused a very severe atrophy of the testes (loss of weight 50-80%). Histological sections of the testes showed impaired spermatogenesis, arrested at the stage of spermatids or mitotic secondary spermatocytes. In rare cases spermatozoa appeared in small numbers and Leydig cells ranged between 3-15 per triangle with an average of 7, while in controls they ranged between 6-20 with an average of 12 (Fig. 4).

While the atrophy of the testes could be the result of diminished secretion of FSH, LH, it could also be due to increased prolactin secretion, which has an antigonadal effect, or to both.

D. Gonadotropin excretion in urine of menopausal women. The gonadotropin content of the urine of 5 female patients, aged between 45-55 years, who were to receive reserpine treatment, was established. Two had 100 RU/L and one 50 RU/L. The remaining 2 had less than 50 RU/L. They received 5-15 mg reserpine daily over 40 days. Their gonadotropin excretion decreased during treatment to 25 RU/L and remained low for 3 months.

Discussion. Whereas reserpine had a relative antigonadotropic effect on male, female, infantile and adult rats, it brought about complete aspermatogenesis and up to 80% atrophy of the testes in pigeons. The severity of the effect of reserpine in pigeons might be due to simultaneous depression of FSH and LH secretion and increased prolactin secretion and discharge, since the latter has an antigonadal effect in pigeons(15,16).

Although administration of reserpine resulted in loss of weight due to decreased food intake, this could not be the cause of the disturbance in the pituitary-gonadal axis, as partial starvation for 3 months did not bring about any of the changes observed.

Other investigators(17) found that ovulation and pregnancy in rats are disturbed by reserpine treatment. Electroencephalographic examination showed that administration of reserpine or chlorpromazine to adult rats before 2 p. m. on day of proestrus inhibited the typical ovulation waves and ovulation (18).

We have reported our hypothesis for the mechanism of increased prolactin production and secretion for chlorpromazine(2,5). This probably also holds true for reserpine. Following suppression of the hypothalamus, a secondary impairment of pituitary and peripheral glands will occur. With regard to part of the pituitary tropins, this suppression becomes balanced according to the push and pull principle, which applies readily to the tropins producing peripheral hormones. As prolactin (LTH) produces milk only, an overshoot of this tropin may occur. This would classify it as an unbalanced tropin, as distinct from other, balanced tropins, which are held in abeyance by a feed-back mechanism of their own peripheral hormone production.

Summary. Reserpine at a dose level of 0.2 mg/kg delayed vaginal opening in infantile female rats, and postponed estrus in normally cycling female rats. In women, vaginal smears likewise indicated decreased estrogen

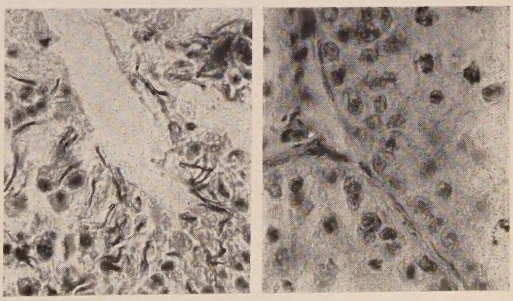

FIG. 4. Histological section of testes (380 ×). Left, normal male pigeon; right, reserpine treated male pigeon. Note absence of sperms in latter.

production throughout treatment. Histological sections of the ovaries of adult rats treated with reserpine showed absence of mature follicles (control 5-8), and presence of corpora lutea, similar to those seen during lactation. Sections of uteri showed the same pattern as in lactating rats. Reserpine delayed testicular descent in young infantile rats. In mature male rats, high doses resulted in regressive atrophy of testes, seminal vesicles and prostates. Testes of pigeons treated with reserpine weighed 20-50% of the weight of controls. Histological sections showed arrest of spermatogenesis at the state of spermatids or mitotic secondary spermatocytes. Only in rare cases were spermatozoa found in relatively small numbers. The gonadotropin level in menopausal women who received reserpine treatment in doses as high as 5-15 mg daily, decreased from 100 RU/L to 25 RU/L. It is concluded that reserpine interferes with the pituitary-gonadal axis by suppression of gonadotropin secretion from the pituitary, apparently as a result of its direct action on the hypothalamus.

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